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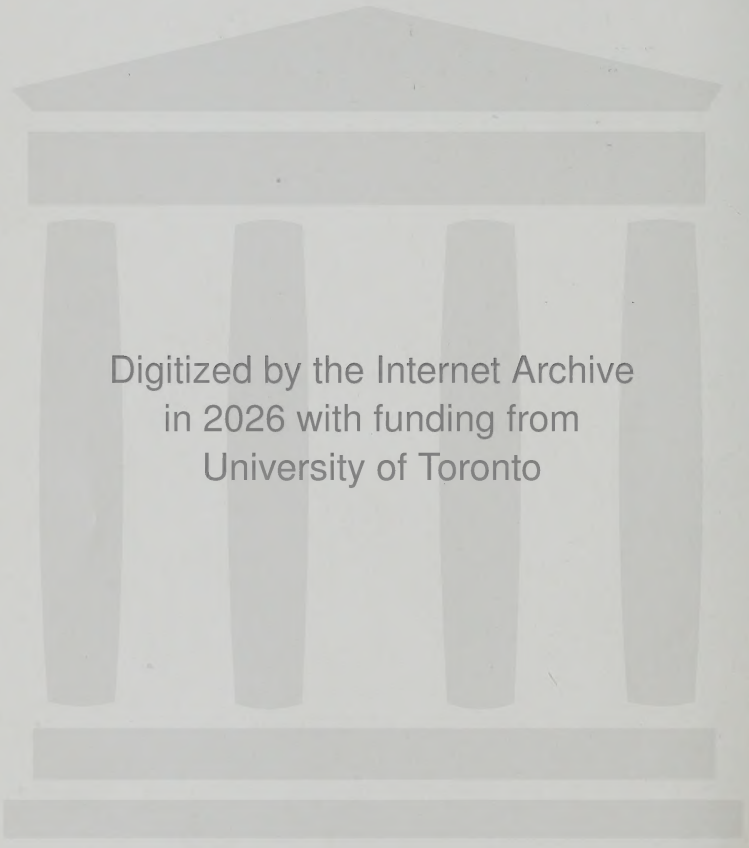
THE STUDENT'S MANUAL OF MICROSCOPIC TECHNIQUE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THE STUDENT'S MANUAL OF MICROSCOPIC TECHNIQUE

WITH

INSTRUCTIONS FOR PHOTOMICROGRAPHY

With 79 illustrations

BY

J. CARROLL TOBIAS

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Preface

In compiling this volume on Microscopic Technique the author has directed his efforts toward producing a book that will fill a real need in its particular field. There are a number of excellent books on the market dealing with the revelations of the microscope, but there are very few that tell the beginning student what to do with material to bring it into proper condition for microscopical examination. Practically no material can be studied to advantage in its natural state, but it must be prepared by a series of chemical or physical treatments to bring it into condition for study.

The instructions given here are adequate for the use even of the student who is just starting out upon his excursions into the world of microscopical things, while comprehensive enough for rather advanced students. Every effort has been made to include the most modern methods of preparing a large variety of materials, at the same time phrasing the matter in such a way that it can be easily understood. The bibliography mentions a number of advanced textbooks to which the student may refer for more detailed information than the scope of this work permits. The main thought back of this book is to include a large variety of materials and objects from which the student may learn the correct methods of preparation before going into fields that are entirely new to him.

The working methods described here have been tested in the author's laboratory, using the equipment described

and illustrated in Chapter XI. Therefore the workability of each process has been thoroughly demonstrated.

The writer can well remember his own flounderings in this field before he learned correct procedure. When he undertook his first examinations with the microscope he was living in a small town with a very limited library. The local public school principal, who had given him his microscope, took an active interest, but was handicapped because he did not know very much about the subject. What he did know was freely given and gratefully received, but had there been such a book as this available then it would have been of inestimable value.

Microscopy is a most absorbing hobby, with so many ramifications that it never becomes boresome. Unless the student is possessed of more than the usual amount of perseverance, however, he is very apt to become discouraged in a short time, due to failure to perceive in his subjects the things the books say are there. Most of the material published today concerning microscopy is reliable, and the mounts may be depended upon to reveal the described features if they are properly prepared.

If this book helps the beginning student to improve his technique to the point where he can prepare workmanlike slides; if it gives him inspiration to continue his studies and if it gives him greater enjoyment of his hobby through increased knowledge of how to do the right thing in the right way, the purpose of the book will be served.

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Introduction

The possessor of a microscope is wealthy beyond the wildest dreams of avarice, for under the lens of his magical instrument he may visit kingdoms beyond the sight of less fortunate individuals. He may peer into the interior of insects and examine the various processes of nutrition, circulation and respiration. He may place upon the microscope slide a drop of pond water and gaze in amazement at the teeming life revealed there. He may explore the inside of a twig or stem and marvel at the precise regularity with which nature has constructed it. He may delve into the past by examining fossil remains that give clues to the life that existed on this planet before the age of man. In short, no object of nature can be examined under the microscope without revealing a sight never dreamed of, beauties unsurpassed by anything to which the unaided eye is accustomed, secrets of nature impossible to comprehend by any other means.

The enjoyment of these beauties need not be expensive. There are on the market a number of small microscopes, really good instruments, that cost comparatively little money. True, these microscopes are not extremely powerful, nor need they be, for most of the work to be dealt with in this book will require comparatively low magnifications. Most beginners in microscopy make the mistake of using powers that are too high and, as a result, they comprehend very little of what they see. Magnifications of 50, 75, 100, 200, 350 and 500 times will answer for most purposes. More to be sought in a micro-

scope than magnifying power is resolving power, which is the ability of a lens to separate fine details and make them visible. This is a variable quality which depends upon several factors, as will be explained later. Of course, if the student has the money to spend he should by all means buy a professional microscope, for the various attachments and accessories which may be secured for such an instrument enormously increase its field of usefulness. But he should not let the lack of such a microscope deter him from the enjoyment to be found in the use of a less pretentious instrument. The author's first work was done with an old school microscope which was limited to 150 diameters, but the optical system was good and it provided endless hours of pleasant recreation.

In addition to the microscope, other apparatus and equipment will be required. This is to be used in the collection, preparation and mounting of subjects for examination, as well as for viewing and photographing them. Most accessories can and should be made at home by the student, not only to save money, but also because the full enjoyment of any hobby comes only to him who does as much of the incidental work as possible. Those of us who really get pleasure out of our microscopes are those who take up the work as a hobby. It is not a business, hence we should not spend money on it as though it were. Make as much of your equipment as your ability and facilities permit. A hobby is an interest to which we can turn for relaxation. It is the expression of a natural desire to be doing something different from our regular work. For genuine relaxation it must be something interesting, and, to make it enduring, it must be active, active as distinguished from static, such as merely collecting things.

What could be a more active hobby than microscopy! Embracing every phase of nature, material for examination is always present everywhere. New accessories may be added from time to time. If one line of study loses in interest, there are others waiting to be investigated, each revealing new aspects, each worthy of attention, and all marvelous beyond words.

To tell the student in the microscopic field where to go for materials, what to do with them when he has them, and what the magnifying power of the microscope reveals to him, is the purpose of this book. It does not pretend to be a complete text book of microscopy, but merely a guide to set the wandering footsteps of the student on the right path, so that he may proceed with greater intelligence toward the selection of those branches of a broad subject that seem to him most interesting. The principal reason for the book is to describe the methods of photomicrography. Before a picture of a specimen may be made, however, the slide must be prepared, so it is necessary to describe the methods by which material is prepared for the microscope. Also, in order to make the student familiar with the technique of photomicroscopy, a chapter has been included which deals with the optics, care and adjustment of the microscope, as well as a short discussion of light, the theory of which, it is hoped, will clarify certain mistaken ideas about magnification.

Chapter I

The Microscope

Optics of the Microscope — The microscope is an optical instrument designed for the purpose of enlarging details to such an extent that they may be clearly discerned by the eye. It may be simple or compound, depending upon whether it contains one or more lenses. A simple microscope is usually found in the form of one double convex lens and is commonly called a magnifying glass. The compound microscope differs from this in that it has several lenses, each magnifying the image of the other until great enlargement is secured. It consists essentially of one lens, the objective, close to the subject, which forms an image which is in turn magnified by another lens, the ocular or eyepiece. Reference to Fig. 1 will help to make this clear.

In this diagram the objective O forms an image of the specimen on the slide S, at the plane P_1. This image, if allowed to reach the eye, would be inverted and a real image. Before it can be formed, however, the light rays encounter the lower lens of the eyepiece B which, in combination with the upper lens C, produces a magnified virtual image at the plane P_2, corresponding to the real image P_1. Thus the magnifying power is the product of the separate magnifying powers of the two lens systems, or that of the objective multiplied by that of the eyepiece.

Thus it would appear that any magnification desired, however great, could be secured simply by increasing the magnifying power of the two lens systems. It would seem at first sight that there is no limit to the amount of detail

we could perceive, for could we not use another microscope to magnify the image produced by the first and thus secure unlimited magnification? Magnification yes, but detail no, for, unfortunately, the amount of detail which may be discerned is limited by optical laws. Mere magnification of the subject does not enable us to see more detail. This quality, known as resolving power, is determined by the construction of the objective.

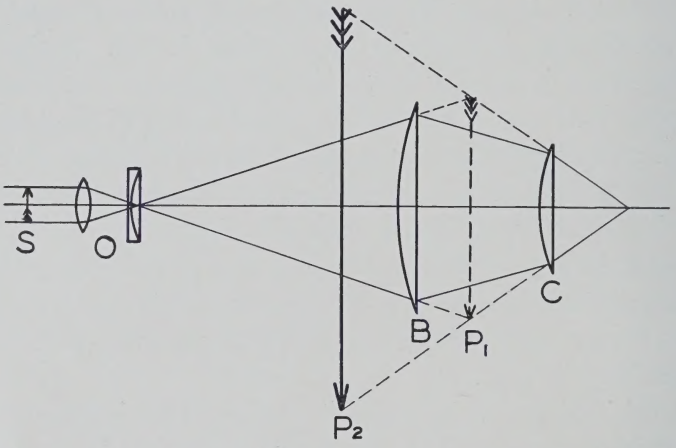


Fig. 1. Diagram of the light path through a microscope.

An eyepiece with a magnification of ten times, that is, one which magnifies the objective image ten times, will give about all the detail that the objective is capable of resolving. This limit of resolving power is fixed by the nature of light itself. Light is not a continuous flow of substance. It consists of definite waves of definite wavelength. This gives to light, in a manner of speaking, a certain structure which makes it impossible to see things that are smaller than the structure of light itself. Resolving power may be defined as the distance by which two small elements in an object must be separated in

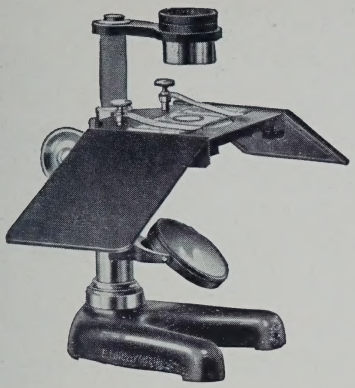


Fig. 2. Leitz Dissecting Microscope.

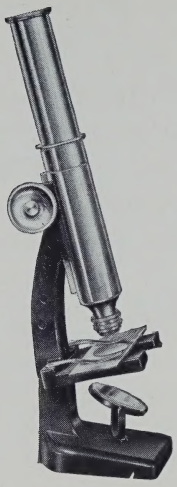


Fig. 3. Bausch & Lomb Amateur Microscope. X75 to X300

Fig. 4. Wollensak Microscope, X235

Fig. 5. Goerz Lomara Microscope.

order to be visible, and is a function of what is known as the numerical aperture of the lens.

In microscopical writings the term numerical aperture is abbreviated to N.A. The higher the N.A. the greater the resolving power and the finer the detail which is revealed. Numerical aperture is equal to the effective aperture of the back lens of the objective divided by twice the equivalent focus. Thus if a very narrow pencil of light is used for illumination, the finest detail which may be revealed by a microscope of sufficient magnification is equal to $\frac{wl}{N.A.}$ in which wl is the wavelength of the light used for illumination. As the pencil of light becomes wider, the resolving power is increased until a maximum is reached when the whole aperture is filled with light. In this case the resolving power is twice as great, as represented by the formula $\frac{wl}{2\ N.A.}$. This same limit is reached when a narrow pencil of light enters the lens as obliquely as possible. The wavelength of light may be taken as one half of 1/1,000 of a millimeter, or about 1/50,000 of an inch. If then we assume a lens in which the effective aperture of the back lens is equal to the equivalent focus, the lens will have an N.A. of 0.5. This lens can separate lines which are 1/25,000 of an inch apart if the back lens is filled with light, but if a narrow pencil is used the lines must be only 1/12,500 of an inch apart to be resolved by this objective. So we see that extremely high resolving power requires objectives of wide numerical aperture, in the order of 1.0 N.A., which will resolve 50,000 lines to the inch. Use of such objectives calls for special equipment and manipulation.

In using a microscope we look through a sheet of glass, the cover glass over the specimen. While this is transparent it may act also as a reflector if the light passing

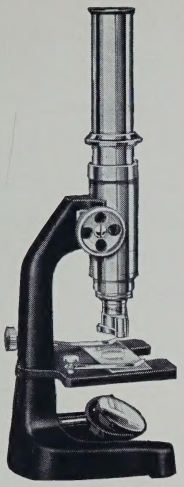


Fig. 6. Leitz SPX Microscope.

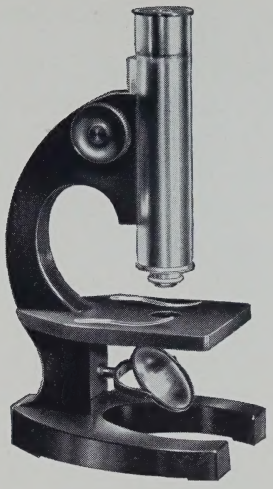


Fig. 7. Bausch & Lomb Amateur Microscope. Magnifying Power X450

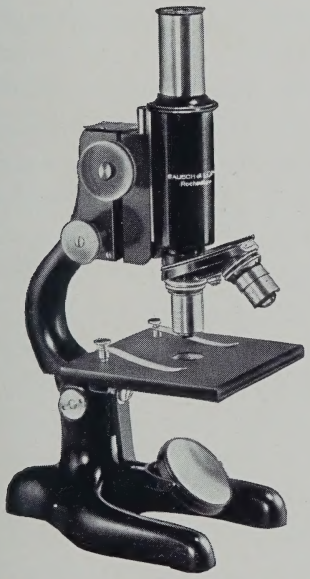


Fig. 8. Bausch & Lomb Standard Microscope stand for high school and college student use.

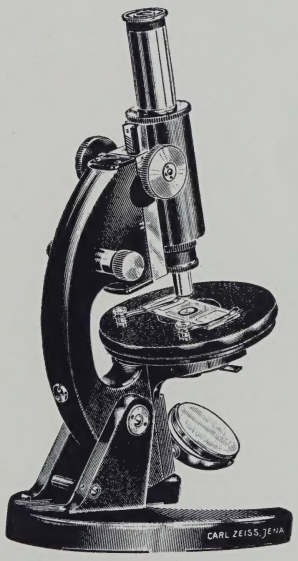


Fig. 9. Zeiss M446 Microscope.

through it strikes it at an angle greater than a certain fixed angle. For the same reason, and more readily because of the black background provided by the inside of the microscope tube, the lens of the objective may become a reflector. In order to control this angle and provide a definite path for the light to travel we equip the microscope with a condenser (the substage condenser) placed in the path of the light. Thus we may control the light and regulate the amount so that it just fills the rear element of the objective when it is examined with the eyepiece removed from the tube. We also place a drop of oil on the cover glass and immerse the objective in this. The oil, having the same refractive index as the glass, presents a homogeneous material through which the light may travel in its own medium, thereby preventing the dispersion which would otherwise take place. Such lenses are known as oil-immersion lenses and by their use numerical apertures as high as 1.4 may be attained, which, under the most favorable conditions, permit resolutions of the order of 100,000 lines to the inch. These objectives are available only on the most expensive professional microscopes, so the beginner need not search for them as accessory equipment to amateur instruments. This digression into numerical aperture is included solely for the fortunate possessors of more pretentious microscopes in order to clear up the terms used in catalog descriptions of such equipment.

Care of the Microscope—A microscope should be treated with the same care as would be given to any other optical instrument. While not in use it must be kept clean, either by keeping it in the original box or under a protecting cover of some sort. If the box is not used the microscope may be protected from dust by placing it

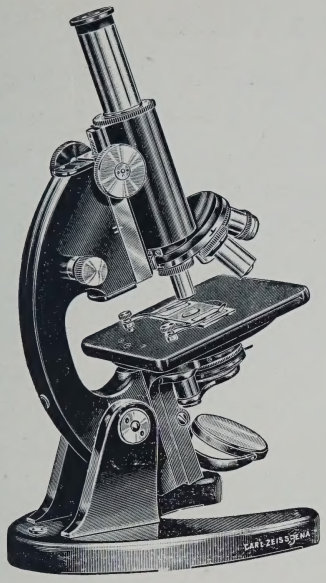


Fig. 10. Zeiss M436 Microscope.

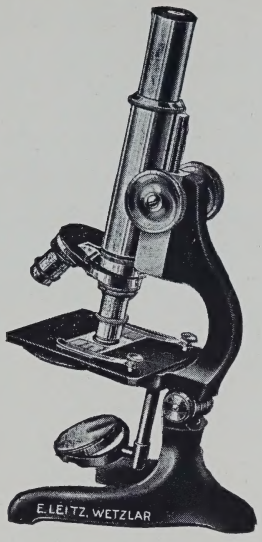


Fig. 11. Leitz O Microscope.

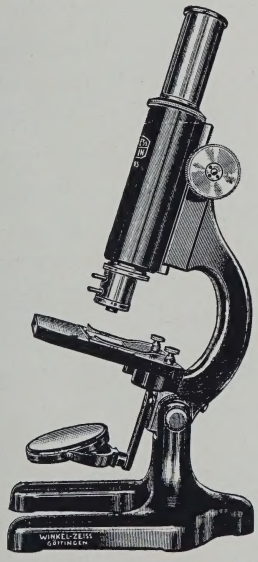


Fig. 12. Zeiss M445 Microscope.

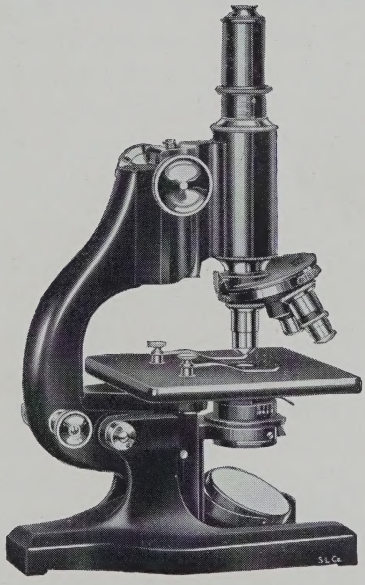


Fig. 13. Spencer 30 Microscope.

under a bell jar or a large battery jar. Any chemical supply house can furnish a jar suitable for the purpose, or possibly one may be found around the house which will serve. In any event the microscope should not be allowed to stand unprotected in the dusty atmosphere of the home or workshop.

If the microscope has been neglected and has accumulated dust, use a soft camels-hair brush and a piece of chamois to clean it. Be extremely careful not to scratch the lenses. If they are merely dusty, wipe the dust away with the brush. If there are drops of water or chemical on the lenses, breathe on them lightly to deposit a thin film of condensed moisture and then remove this with Japanese tissue. This is a soft, light, vegetable tissue made in Japan which was first suggested for use by microscopists by Prof. S. H. Gage of Cornell University. It is universally used today by microscopists to clean the objectives after immersion in oil or water. Photographers also use this tissue to clean photographic lenses and it may be purchased from any photo-supply house. If the objective lens should become soiled with a substance such as Canada balsam, it should be cleaned immediately as well as possible by simply wiping with a piece of dry tissue to remove the greater part of the balsam, then finished with a well-washed linen rag moistened with alcohol. Note that the rag is to be only moist, not wet, and the glass must be wiped at once with a dry part of the rag, followed by the breath and Japanese tissue. If the eyepiece of your microscope consists of two lenses do not take them apart. This may be possible, but serves no good purpose and affords a splendid opportunity for dust to get into the tube and make all sorts of trouble. Keep the lenses clean but do not overdo the cleaning. Optical

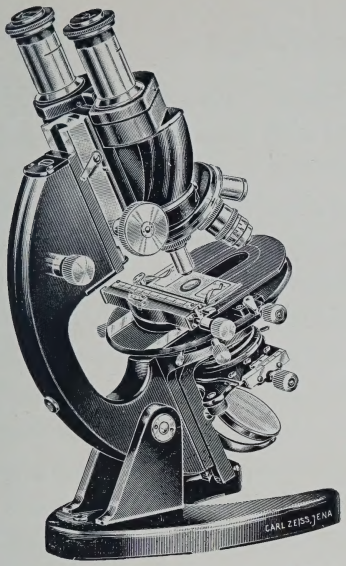


Fig. 14. Zeiss M317–4 Microscope.

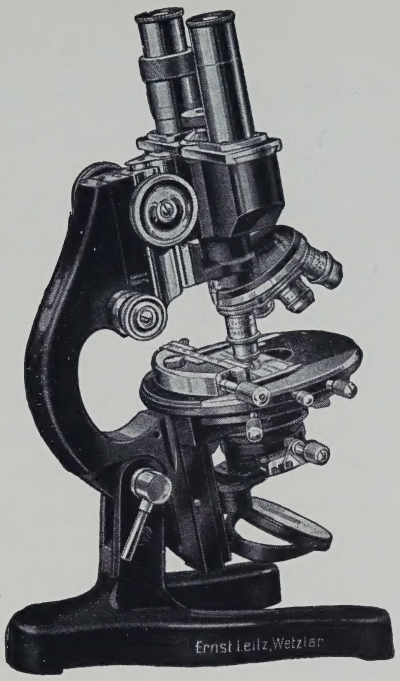


Fig. 15. Leitz ABM Microscope.

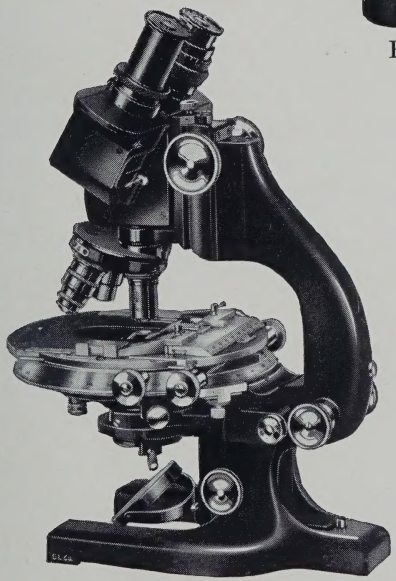


Fig. 16. Spencer 5 Microscope.

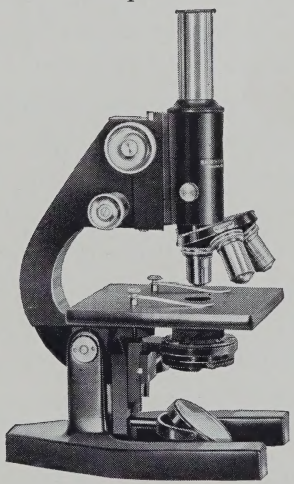


Fig. 17. Bausch & Lomb Laboratory Research Model Microscope. Triple objective, substage condenser and extra large stage.

glass is soft and easily scratched and a scratched lens will never reveal the full beauty of the preparations to be examined.

While it may seem not worth mentioning, it is surprising to learn that a great many people do not know how to focus a microscope. The author has seen many students look through the tube and focus *down* on the subject. This is reprehensible. Never do it, for sooner or later you are going to run the objective down too far and break the cover glass and possibly ruin the slide, or you may break the objective, which would be even more regrettable. The proper way to bring an object into focus is to focus *up* on it. To do this, look at the objective from the outside, at the same time lowering the tube toward the specimen until you are sure it is closer than is necessary to secure sharp focus. Then look into the tube and draw the lens *away* from the subject until it is in focus. A good rule to remember is this: always move the tube down when you are looking *at* it and up when you are looking *through* it. If the microscope is equipped with both coarse and fine adjustments the tube may be lowered with the coarse adjustment knob, then roughly focused by raising the tube and finally getting critical focus with the fine adjustment.

Never try to examine an object that is not in perfect focus, for this causes eye strain. Be sure the focus is the best possible by making slight adjustments with the fine adjustment knob. Run the tube down a little distance and if the image appears to be improved the first focus was not correct. If there is no apparent change in appearance the focus was probably just about right and so should be restored.

Another error of many students is the use of too much

light. More detail can be seen when the field is moderately lighted than when the eye is blinded by a dazzling glare. Just as the strong sun has a blinding effect when we look into it, so the light projected through the microscope affects the eye. Too much light is objectionable because it may lead to serious eye injury. On the other hand, do not go to the other extreme and try to work with not enough light, for this is just as bad, since we are forcing the eyes to work in semi-darkness. Keep the field sufficiently lighted to be pleasant to the sight and no injurious results will be felt. If your microscope is fitted with a diaphragm, stop this down until there is just enough light to see the object clearly. If the object is thick or opaque, more light will be needed, in which case open the diaphragm. If this accessory is not available, as

Fig. 18. Leitz BI–D Microscope.

in the smaller microscopes, reduce the intensity of the light by tilting the mirror, or by moving the illuminator away from the microscope, by placing sheets of tissue

paper in the light path or by reducing the size of bulb used.

Setting up the Microscope — When an object is to be examined, the microscope is placed near a window, or, if at night, near a lamp of some sort from which light may be picked up by the mirror and reflected up through the object. The tube is inclined at a convenient angle and the mirror is manipulated until the light is reflected upon the slide where it may be seen. The eye is then applied to the eyepiece and the illumination examined. It will probably be imperfect or unevenly distributed, in which case the mirror must be adjusted until the entire field is illuminated. Now remove the eyepiece from the tube (if this is so arranged that it may be done without unscrewing it) and examine the little spot of light which appears upon the rear lens of the objective. If this spot is not in the exact center of the lens, shift the mirror until it is, for this is the position for the best illumination. The secret of securing this condition lies in picking up the light in the center of the mirror. If artificial light is used, move the illuminator until the image of the bulb or other illuminant is in the exact center of the mirror, then direct the light upwards through the stage opening into the microscope. Now lower the tube, focus upward until sharp focus is secured and the examination may proceed.

Many objects may be examined in a dry state simply by placing them on a slide for viewing. Others, such as the minute organisms found in pond water, may be examined only in their natural state, for as yet no method has been discovered or invented for mounting many of them. Other subjects require preparation for mounting, either by washing, slicing, decoloring, softening, staining or any

one or combination of several processes. Indeed, the preparation of the collected material is just as fascinating as the examination of the prepared slides, and the student should take pains at the very start to learn the correct ways of preparing material for examination. This preparation by the student is not only desirable, but really necessary if he wishes to learn something of the wonders of nature. Dealers' lists contain large numbers of slides ready made, but for the student to purchase a slide containing the foot of a fly is foolish and extravagant. If he lives in some inaccessible place and has no idea what a slide is he might buy one to show what is to be aimed at in preparation, but for no other reason. Certain very rare slides may be purchased but there are so many thousands of subjects waiting to be taken home and mounted that he could fill his days for a year and do no more than scratch the surface. Methods of preparation and mounting will be described later in the text. The student may begin at once his work of collecting a library of microscopic material.

Chapter II

Microscopic Objects from Water

The main sources of microscopic material may be divided into three classes, namely, the waters of ponds, lakes and streams, animal life, and vegetable life. These, with their various side branches, afford an endless variety of material. One of the most interesting of these groups is the first, ordinary water that has collected in a small or a large body, either still or moving. Here are to be found microscopic creatures by the million, in endless variety, some animal, others vegetable. Hours of study and observation may be expended upon one drop of water from a pail into which a handful of straw has been thrown and left to stand for several weeks. Should this source not be prolific enough to suit the fancy, take a small jar and run it along the trunk of a tree submerged in a lake. Put a drop of this water on a slide, magnify it fifty times and you will see creatures tumbling over one another, creatures you never thought existed. If you are still not satisfied, take the small jar again and gently scrape it along the bottom of a shallow portion of a lake or stream. Be very careful not to collect too much mud, but let it just skim along the surface. If there are any aquatic plants growing there, scrape their stems and the under sides of the leaves with your collecting bottle. Put a drop of this collection on your slide and be prepared for a shock. You have now collected an entirely new lot of animalcules, thousands of them, and yet you have seen but a very small portion of what you may find. Almost every drop of water you examine will contain new forms of life. These

forms vary with the seasons, the location and the character of the water. Certain forms live only in fresh water, others in salt water, still others may be found only in places where there is a mixture of the two, such as at the mouth of rivers.

Since water is such a prolific source of microscopic life many beginners turn to it for their first material, so we will describe a few collecting tools which will make the work easier and more interesting.

While a bottle or jar may be used for collecting microscopic material from shallow pools and streams, it is not the most desirable device because it permits the collection of too much water along with the organisms. We therefore make a collecting net which permits the water to drain away while the aquatic life is concentrated in the bottle at the apex of the net.

To the end of a broom handle three or four feet long attach a ring made of heavy galvanized iron wire about eight inches in diameter. This may be fastened by drilling a hole in the end of the wood and inserting the twisted ends of the loop, or it may be wired to the handle with thin copper wire. To this loop fasten a conical net of muslin about twelve inches long. The apex of this cone should be hemmed to prevent fraying. Into the hole left in the apex insert a small wide-mouth bottle of about four ounces capacity, holding it with stout rubber bands. This permits removal of the bottle when it is filled. To use the net simply drag it through the water, skimming the bottom, scraping the stems and leaves of aquatic plants, scraping the trunks of submerged trees, piling or anything else that may be in the water. When the small bottle is filled with the concentrated collection it may be emptied into a larger bottle containing a quantity of

water. In this way samples may be taken from a number of places, thereby increasing the number of species of animal and vegetable life collected.

One drop of this water examined under the microscope will reveal an enormous quantity of living things. These need air to support life and should not be confined in a tightly closed jar or bottle. The forms of life are tiny but there are thousands of them and they use up air at a surprising rate, so leave a generous amount of air in the container in which you carry home your collection. These specimens must also be fed if they are to be kept alive for future study. This is not difficult to do as any aquarium affords an excellent breeding place. The aquarium must be kept for just this purpose and should not contain fish. Let the student look up the construction of a balanced aquarium and build one along the lines suggested. Introduced into this aquarium the microscopic animals will live and flourish and be always at hand when wanted for study.

Now to get back to the examination of the material gathered with the collecting net. This will contain a variety of things, all new to the student. It is difficult to say just what has been collected since each locality produces its own species, and many that are found abundantly in one place are entirely absent in another. However, it is fairly safe to say that the greater portion of the forms present will consist of diatoms, desmids and algae. There will doubtless be some Rhizopods, a good many Infusoria, probably some worms, numerous Rotifera and some Polyzoa.

To classify all of these forms would be impossible in the space allotted to this portion of microscopic examination, so with a few descriptions and illustrations, we will

pass on to the methods of examining and preparing certain of the subjects for examination.

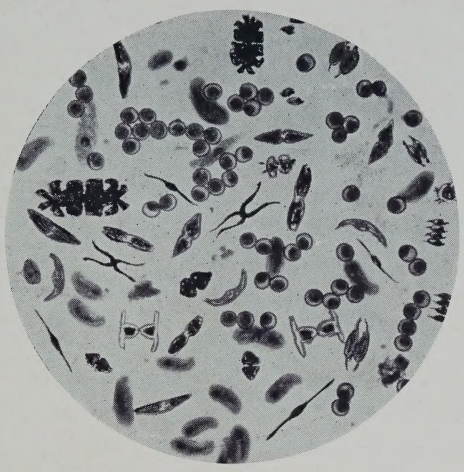


Fig. 19. A group of desmids from an aquarium culture. X600

The desmids and the diatoms are two closely related groups of aquatic plants. Some difficulty will be experienced in distinguishing one from the other, but after a little study the differences will become apparent.

Desmids are usually found in the sweetest and freshest water. Salty or brackish water contains none at all, while diatoms flourish there as well as in a mill-pond. Living desmids are always green, diatoms are always brown. There are other means of identification, such as flattening the soft cell wall of a desmid by pressing the cover glass down against the slide, and rupturing the cell wall. The green coloring matter (chlorophyl) and the colorless protoplasm that fills the cell may then be forced out. The cell wall of a diatom is hard and brittle, being composed of silicate. The cover glass may be pressed down on a diatom until the glass breaks without flattening or

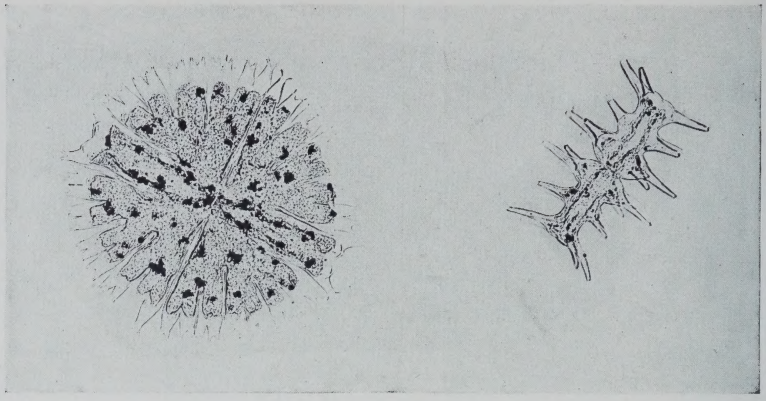


Fig. 20. Desmids. Two species of the Genus Micrasterias. X185. Slides by J. M. Furber. Photographs by Irving L. Shaw.

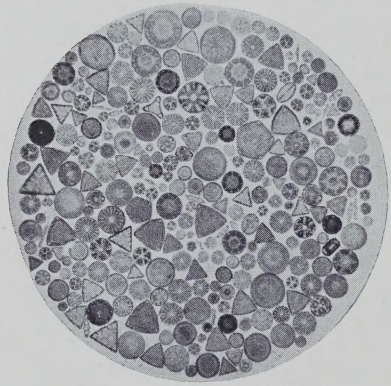


Fig. 21. A group of diatoms. After Beavis. X35

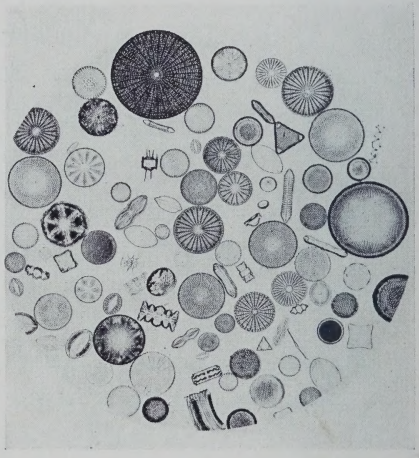


Fig. 22. Diatoms. Type-slide by Chr. Michelson, Odense, Denmark. X78. Photograph by Irving L. Shaw.

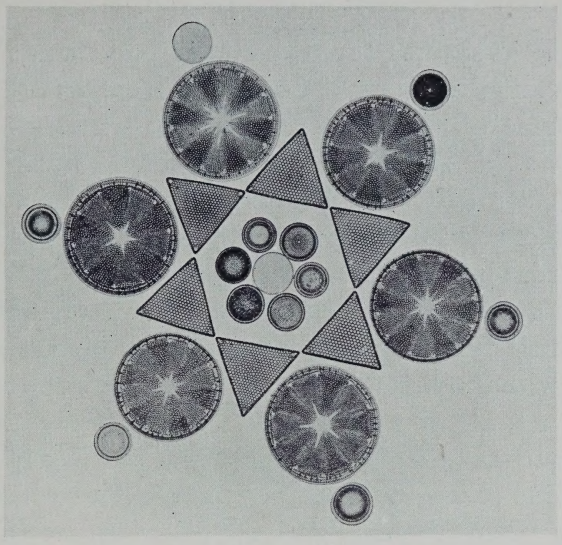


Fig. 23. Group of arranged diatoms. X60. Photograph by Irving L. Shaw.

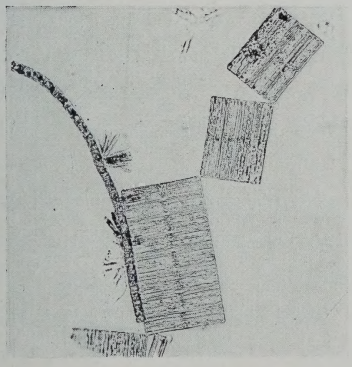


Fig. 24. Living diatoms from a water trough. X180. Slide and photograph by Irving L. Shaw.

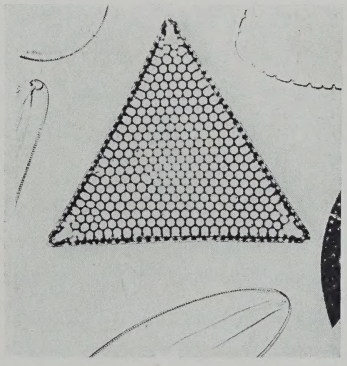


Fig. 25. A Diatom, Triceratrum. Slide by P. Klarsen, Odense, Denmark. X265. Photograph by Irving L. Shaw.

changing its shape. It may roll over and change its position, presenting an aspect quite different from that first seen, but it will probably roll back again and appear as it did at first. If the cell wall is fractured the break will not be irregular or of the appearance presented by a soft wall when broken, but will display the characteristic fracture of a hard, glass-like substance.

Both desmids and diatoms have the power of locomotion, frequently moving from place to place. When mixed with mud, as they probably will be when collected with a net, desmids slowly work themselves free and rise to the surface where they collect in a green scum or line at the side of the vessel nearest the window, whence they may be taken for examination. Diatoms have a similar power of motion, but they usually move more rapidly. Under the microscope the desmids may be seen moving slowly and sedately across the field in a straight line. The diatoms start across the field, get half way, then stop and retreat or go in an entirely different direction. They always seem to have important business to do and to be in a tremendous hurry to get it done. Thus an object that may seem to be either a diatom or a desmid is not a diatom if it moves slowly, nor is it a desmid if it darts around the field like a humming bird.

Rhizopods, while not a large group, are interesting because they are lowest in the scale of animal life. Some are entirely without body protection of any kind, having neither skeleton nor shell, the body consisting merely of a soft jellylike mass of protoplasm. Yet they move about, live and reproduce their kind. The most familiar member of this family is the amoeba. This animal moves by protruding a portion of its body to form a sort of arm, then elongating the entire body and finally contracting

forward until it has resumed its former shape. This protrusion may take place from any part of the body, for it has no organs of locomotion as we understand them. When the animal feeds it literally surrounds its food. Having no stomach, digestive organs or alimentary canal, it simply wraps itself about the food particle and somehow digests and converts it into protoplasm. It reproduces asexually, that is, by a sort of budding. A protuberance shows itself which gradually grows until it

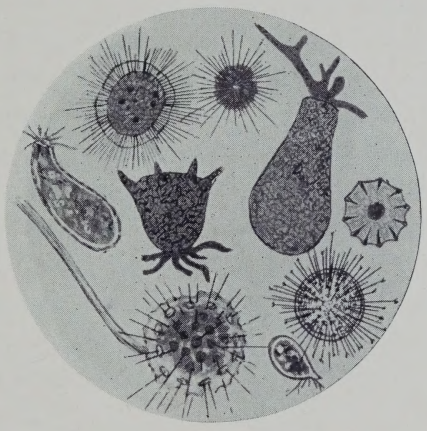


Fig. 26. A group of rhizopods.
From a drawing by the author.
Approximately X300

attains some size, then parts from the parent and goes away to live its own life.

Others of the rhizopods are slightly more advanced, especially the members of the genus Difflugia which build for themselves shells of sand grains, cemented together in a perfectly regular form with each grain fitted into its place. If sand is scarce it will use diatom shells to construct its own shell, often taking those which are longer than itself, attaching them lengthwise, side by side

and parallel to one another. Still another form, the most beautiful of all the fresh water rhizopods, lifts itself on a long stem and surrounds its body with a hollow latticed sphere through the openings of which the pseudopods (false feet) are extended in search of food. Any small animal or vegetable substance supplies the rhizopods with food. Diatoms, desmids, Infusoria or anything small enough to be seized is grasped by a pseudopodium, surrounded by protoplasm and digested.

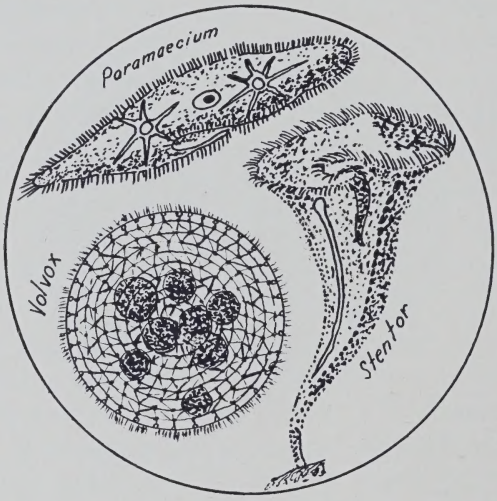


Fig. 27. Three familiar Infusoria found in pond water. From a drawing by the author.

The Infusoria group contains many of the microscopic objects familiar to us from high school study of biology, such as Vorticella, Stentor and Paramecium. The group derives its name from the fact that many of the individuals were first discovered in infusions, that is, water in which animal or vegetable matter had been steeped and was decaying. Since their first discovery the animals

have been found in great variety and abundance in even the sweetest water, although they abound in incredible numbers in stagnant pools.

The best way to procure specimens is to place the dip-net under the mass of aquatic plants upon which they are found and lift the mass with the net. If the plant is lifted from the water, the water draining away will carry with it many of the specimens, which are thus lost. A bottle may be placed under the plant and the leaves and stems scraped with the bottle rim, when the Infusoria will be carried into the bottle. Some of them are free-swimming, others are permanently attached to water plants, while a third group builds shells like those of the rhizopods. The free-swimming varieties may be easily collected and placed upon the slide for examination, while those permanently attached may be found only by cutting away a part of the plant and examining it. Some of the most interesting types are firmly adherent to the water plant Utricularia and to other plants with finely divided leaves, every part of which should be searched with the microscope, especially the forks of the leaves.

Those that build cases secrete a sticky substance to which extraneous floating matter is pretty sure to adhere, building up a protective covering which surrounds the animal. These cases or loricae are built of material carried toward the animal by motion set up in the water by organs with which it is provided. These organs serve to create a current of water which carries food to the animal, for being immobile it cannot move about in search of food.

One very beautiful form of Infusorium representing the fixed type is the species of Carchesium, illustrated in Fig. 28. The single stem, which is attached to some

submerged object, divides at the summit into a large number of branches, each one bearing at its end a tiny bell-shaped Infusorium, while numerous other individuals are disposed along the stems on branchlets of their

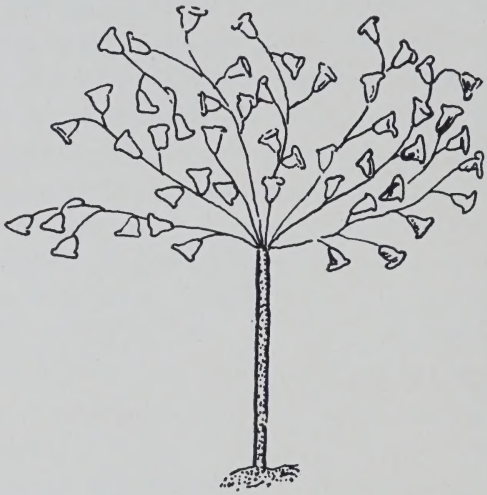


Fig. 28. Carchesium. A fixed form of Infusoria. From a drawing by the author.

own. Many of the fixed Infusoria are equipped with muscles that enable them to contract when alarmed, of which Carchesium is a good example. Running through the stem and all of the branches is a cord-like muscular thread which contracts when the animal is stimulated, pulling the entire colony, which is colorless and may contain as many as a hundred individuals, toward the point of attachment. This contractile property is elective, for one branch at a time may be affected without disturbing the others, or the entire colony may contract at once.

All Infusoria are provided with cilia (hairs) or flagella (lash or thread-like appendages), which serve two pur-

poses. They provide a means of locomotion, and by their vibration create a current of water which moves food particles toward the animal. Carchesium is an example of the ciliated type in which the front or large end of the bell is surrounded by a wreath of cilia visible under a high power. When the creature contracts these vibratile hairs fold together and the animal appears like a tiny ball.

Another interesting group of Infusoria are the Vorticellae. There are about seventy species known, but we will describe only one, since the microscopist will have no difficulty in recognizing the genus once he has seen one member. They belong to the class of fixed Infusoria, are very common, scarcely a leaf or twig of any aquatic plant being without at least one of them, and most of them are colorless, or nearly so. Green ones do occur, *Vorticella smaragdina* being one example, but most of them are entirely devoid of color. Individuals are invisible to the naked eye, but magnifications of X250 will reveal them clearly. The body is somewhat bell shaped and is carried at the apex of a contractile stem. The front or rim is wreathed with cilia, for the same purpose as was explained in connection with Carchesium.

While individual Vorticellae are invisible, their multiplication in colonial groups is sometimes so rapid during the summer that the entire colony breaks away from its mooring and goes floating away. These groups look like small spots of saliva or mucus floating on the water or attached to the leaves and stems of plants. When such a spot is discovered and touched with a needle-point, it seems to grow smaller or disappear almost entirely. Pick the plant on which it is found and place it in water for later examination.

Do not be alarmed if, upon reaching home, your colony

seems to have vanished. Place the plant in an aquarium and await developments. It will presently appear and afford many hours of study. These Infusoria can contract with a suddenness that is most disconcerting. The observer may be calmly examining a Vorticella under the microscope when, for no apparent reason, it disappears like a flash, and one feels that the slide has been moved. Soon, however, it may be seen separating itself from its support, the coiled stem growing longer and straighter, until the whole animal is again in view. Sometimes it is barely extended when it again leaps from sight. If the student has an aquarium he will do well to introduce several colonies of these interesting animals, for they are always worth studying.

Several species of Stentor may be found, the most common being *Stentor molymorphus,* illustrated in the drawing (Fig. 27). In shape the bodies are somewhat variable at will, and vary slightly in the same species. The largest are trumpet-shaped and are, as a rule, permanently attached at the narrow end of the body to some fixed support. Some species (*Stentor Barretti*) form a lorica into which they retreat when disturbed, folding the frontal border to form a covering over the lorica. A few are free-swimming by means of cilia and vibratile hairs. In color they may be green, red, blue or almost black.

Paramecium, illustrated in the drawing, is sometimes called the slipper animalcule because of its shape. It is frequently found in ponds but may be easily raised in a tumbler of water to which a few small pieces of hard-cooked egg have been added. Pure cultures of enormous numbers of Paramecia may be thus reared in a few days. On one surface of the animal is an opening that leads to the mouth. The entire body is covered with cilia, while

one species has a caudal tuft of setae (stiff hairs) at the posterior extremity. It reproduces by asexual division. Two contractile vesicles may be seen, one in each half of the body.

Volvox globator, another free-swimming form, is illustrated in the drawing. This beautiful little animal may be found in profusion early in spring, and will multiply freely in an aquarium. It is quite large, in fact large enough to be seen with the naked eye, and swims about freely in a drop of water. The illustration shows an individual containing a number of sporific inclusions that will presently leave the parent cell and develop into new individuals.

With this short introduction to the many beautiful and interesting objects to be found in water, we must leave that subject and go back to the business of preparing the material for examination.

In addition to the collecting net a few more accessories will be required. These, like the net, can be made by the student. The easiest and most efficient way to remove drops of water from a gathering to the slide is with a dipping tube. This is merely a length of glass tubing of small bore drawn out to a point in the flame. Such tubing is obtainable in various diameters at any chemical supply house. Tubing of about three sixteenths to one quarter of an inch is about right. Several dipping tubes should be provided, one straight, just as it is received, two with points of different diameters drawn out in the flame, and two with curved drawn-out points. These tubes are used by holding the moistened finger over one end and immersing the other end in the water. When the finger is removed from the free end the water will rush up into the tube until it reaches the same level as the water on the

outside. In this way a drop or a larger quantity of water may be transferred easily and quickly to the live cage for examination.

The live cage is necessary in the study of aquatic organisms because by its use we can keep the objects alive for a long time and study them in life. The usual practice of students is to drop a cover glass on the specimens and thus restrain their movements and confine them to a limited space. This method, while it works after a fashion, requires constant additions of water to keep the animals alive. This is bothersome and can be avoided by using a live cage or box.

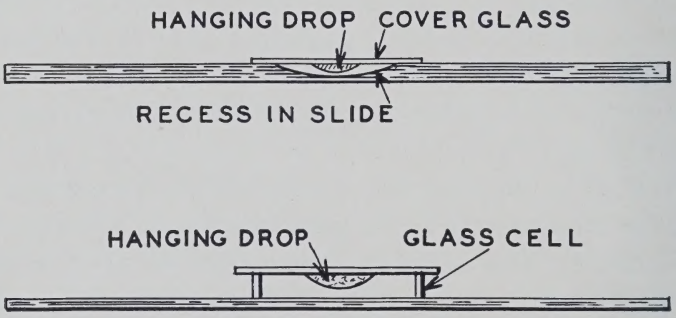


Fig. 29. Diagrammatic views of live boxes. Depression slide and cell slide.

One form illustrated in Fig. 29 is made from a ring of glass, celluloid, metal, wood or cardboard cemented to the slide. The first three materials need no preparation before use, but wood or cardboard rings should be given a coat of shellac to make them waterproof.

Glass tubing of large diameter and thin wall, such as a large test tube, makes an excellent cell. With a triangular file or a glass cutter score two marks on the outside of a test tube all around it, about one quarter of an inch apart. Heat a rod of metal and touch one of the

marks with it. The tube will break off neatly at the mark. Repeat at the other mark and you will have a cylinder of glass one quarter of an inch deep. Now take some Canada balsam and apply a ring of it to a slide, making the ring of the same diameter as the glass cylinder and using a generous quantity. Heat the slide over the spirit lamp until the balsam is nearly hard, then press the warmed glass ring into it and set it aside to harden. When hard take fine sand and water, or emery powder and water, and working on a piece of glass, grind the uncemented rim of the glass ring flat. If desired, the depth of the cell thus formed may be reduced to one eighth of an inch or less by continued grinding. When the desired depth is attained wash the slide and cell in water, dry it and paint a ring of balsam around the outside of the joint and allow to dry thoroughly, when the cell will be ready for use. Such a slide may be used for a hanging drop or by filling the cell with the water to be examined. In either case paint a ring of vaseline on the rim of the cell before applying the cover glass, to prevent evaporation. Rings made of brass tubing may be made up on slides in this same way, or in a pinch a washer may be used.

Illustrated in Fig. 29 is another type of slide that may be used for holding a drop of water. This is known as a depression slide, since it has a shallow depression ground into it. These slides are available from supply houses.

Equipped with a collecting net, dipping tubes and a live box the microscopist has all the equipment needed to afford him hours of entertaining study of pond life. All of the forms mentioned may be found without any trouble, as well as thousands of others that will delight and fascinate. Furthermore, not all of these minute specks of life have been found and described, so there is

ample field for research work in this branch of microscopy. It is just as easy for a student worker to discover new forms as it is for the trained scientist, and every observer should be on the alert for new species.

As mentioned earlier, many forms of aquatic life are difficult to mount permanently on a slide. Others are comparatively simple to mount and the student may want to know how these may be preserved. The greatest difficulty encountered in mounting Infusoria, Rotatoria, Rhizopoda, Hydra, Obelia, etc., is that of killing them in such a way that they remain expanded. Most killing agents stimulate them to such an extent that they immediately contract into a shapeless mass. To prevent this we first apply a reagent that will anesthetize them in an expanded state, then kill them with another reagent, usually combined with a fixing agent to prevent the postmortem changes that take place almost at once.

Many forms may be successfully anesthetized with Epsom salt made up into a saturated solution and added slowly to the water. Separate a small quantity of the water containing the desired forms and slowly add a saturated solution of Epsom salt until the desired effect is secured. Keep the water as quiet as possible to prevent contraction, and add the solution very cautiously. One very good way is to place a piece of string in the end of a dipping tube, drop some of the solution in the tube and suspend this so that the string just dips into the water. In this way the salt solution slowly diffuses into the water without setting up any currents to disturb the animals. Crystals of menthol or chloral hydrate may be placed in a filter and floated on the water to achieve the same result. The author has had considerable success with Vorticella, Stentor, Paramecium, etc., by subjecting them to an atmosphere of formalin, which kills the forms quickly

in an expanded state. The method used is to place the water in a cell-slide in a large dish and leave until all motion in the water has subsided and the forms are fully expanded. A few drops of formalin are then introduced into the dish by means of a previously attached dropping tube and the dish is covered with a plate of glass to confine the vapors. The slide is allowed to remain a few minutes and is then taken out, the water transferred to albumenized slides and these treated as follows.

The slides to be used are first prepared with Mayer's albumen fixative (See Chapter IV). A drop of water containing the desired organisms is then placed on each slide and allowed to become nearly dry, assisted by gently blowing on the drop of water. When nearly dry plunge the slide in 70% alcohol to coagulate the albumen and hold the objects to the slide. Now place the slide in one of the fixing agents (See Chapter III). Shaudinn's solution is the one usually recommended for Infusoria, but Worcester's fluid works just as well. Leave until thoroughly fixed, then wash as instructed for the removal of mercuric chloride fixatives. The washed slide is now stained, using alum-haematoxylin and eosin, or picrocarmine and methyl green according to the directions given in Chapter VI. Following staining, the slide is dehydrated, cleared and mounted in balsam.

Taking advantage of the mildly anesthetic properties of clove oil the writer has recently conducted a series of experiments in which this reagent was used to anesthetize strongly contractile aquatic organisms prior to fixing them. The method was tested on Hydra, Rotatoria, Vorticella and other difficult forms with results almost comparable with those resulting from the use of cocaine and Novocaine. After several techniques were tested the following was adopted as a satisfactory procedure.

Bring the objects from the aquarium or collection into a small watch glass that can be placed on the stage of the microscope for observation. Make the transfer with a dipping tube, including enough depth of water to permit the organisms to expand fully. When a sufficient number of individuals has been transferred to the watch glass, allow the water to become quiet and observe through the microscope. When the forms are fully expanded place a drop of clove oil on the surface of the water, using a pipette. Bring the tip of the pipette close to the water and ease the oil out very gently to avoid disturbing the water and making the organisms contract. The oil film will spread over the water and its anesthetizing action will slowly take effect.

Have ready a pipette full of a suitable fixing fluid, such as Bouin's or Worcester's fluid. Observe the progress of the anesthesia carefully and as soon as all signs of motion cease, add the fixing fluid. It is important that the fixing fluid be added at the earliest possible moment after the forms are quiet. General death of the individual follows closely upon anesthesia and many organisms start to disintegrate immediately after death. It is the purpose of the fixing agent to arrest this disintegration by killing and fixing the organism in one operation. Regulate the proportion of water in the watch glass so that about an equal volume of fixing fluid may be added.

Allow this fluid to act about fifteen minutes, then drain off most of it and add pure fixing fluid. After the objects are thoroughly fixed, they are washed and treated as required by the fixing fluid used (See Chapter III).

Temporary staining may be effected by drawing neutral red stain under the cover glass in the live cage with blotting paper in the manner described in Chapter IV.

Chapter III

Killing, Fixing & Preserving

Living material is of very little use in microscopical study. Accordingly the animal or plant must first be killed so that the elements desired for study may be dissected out. Fresh tissue from recently killed subjects should be selected in all cases. Killing usually refers to the general death of the subject and does not apply strictly to the life processes of individual cells, which continue for some time after the general death of the specimen. It is the purpose of the fixing agent to terminate this life process, which it does effectually only if it is applied before total cessation of vital activity. Destructive post-mortem changes follow almost immediately upon the death of the cells and unless this is arrested by the action of the fixing agent, serious alteration of the tissue takes place. Fixation, then, is the process of terminating cell life and hardening and preserving the tissues in a condition as nearly like that obtaining in life as is possible. The process is effected through the chemical action of certain materials which are accordingly called fixing agents.

Preservation of structural elements to present a faithful impression of their true character is a fundamental requirement in the preparation of microscope material. The subsequent operations of dissecting, staining, sectioning and mounting serve only to make the examination more intelligible by clarifying the material and differentiating structures that have been brought into proper condition by fixing. Considerable thought and

effort must sometimes be expended upon the selection and application of an appropriate fixing agent for the material in hand. Its composition, reaction and application must be such that it will penetrate and kill the tissues quickly to preserve their active characteristics. It must do this with a minimum of alteration in their structure, and it must harden and preserve them enough to prevent post-mortem changes, yet leave them amenable to the subsequent processing by other reagents in the operations of staining, sectioning and mounting.

No one chemical yet discovered possesses all these virtues. A reagent that fixes one class of tissue may act as a macerating agent on another class. Most of the reagents that fix nuclei satisfactorily are known to alter or destroy certain cytoplasmic inclusions. Hence we combine in one mixture a group of reagents to secure a desired effect.

The more generally used fixing agents contain one or more of the following chemicals in varying proportions, alcohol, formalin, acetic acid, mercuric chloride, chromic acid or its metallic salts, picric acid, nitric acid and osmic acid. Many combinations of these, as well as a number of other reagents, have been proposed, tried and discarded. Relatively few of those to be found in microscopical writings are of value to the student. The few that are of value have been selected and described below, together with a short list of the materials for which they are satisfactory. The alert student will be able to select from the formulae given one or more fixing agents to be used on the material with which he is working.

The first fixing agent to be considered is pure methyl alcohol. It is usually used in strengths of 70% to 95%. This is an acceptable fixative for crude work and for robust organisms like insects and crustacea. It is entirely

unsuited to soft, delicate tissues or those containing much water, for it shrinks them badly. It causes little or no chemical alteration, except in the solution of fats and oils. Weak alcohol (30–35%) is useful for fixing tissue that is to be dissociated by maceration. It dissolves intercellular cement substance and fixes cell contents with little shrinking or alteration.

Acetic acid is a weak fixative for some tissues and has a decided tendency to make them swell. Taking advantage of this property we combine it with alcohol in such proportions that the shrinking propensity of the one reagent is balanced by the swelling action of the other, as in the following formula:

CARNOY'S FLUID

Glacial acetic acid	10 ccm
Alcohol (85%)	85 ccm
Chloroform	30 ccm

Mix immediately before use.

This mixture penetrates and fixes with fair speed and is a suitable medium to use on rough work with fairly robust material. When fixation is complete, transfer the material to 80% or 90% alcohol.

Very impermeable material should be fixed in the following mixture, which will penetrate and kill even the most refractory organisms:

CARNOY AND LE BRUN'S FLUID

Alcohol, absolute	50 ccm
Glacial acetic acid	50 ccm
Chloroform	50 ccm

Mix immediately before use.

Saturate the above mixture with mercuric chloride.

Use this fixative only for the most impermeable objects, such as nematodes, and heavy-shelled eggs. When the material is fixed it must be treated with iodine as described under the use of mercuric chloride fixing agents listed further along in this chapter.

The following mixture is very good for insects, crustacea and botanical specimens of many kinds:

FORMALIN-ACETIC-ALCOHOL

Alcohol (85%)	85 ccm
Formalin	10 ccm
Glacial acetic acid	5 ccm

Keep a stock solution of alcohol and formalin and add the acid to measured quantities of this when needed for use. Fixing is complete in from one to twenty-four hours, when the material should be transferred to 85% alcohol for preservation.

Formalin is an important fixative with numerous desirable properties. It does not dissolve carbohydrates or fats, penetrates rapidly, preserves tissues indefinitely without serious alteration, leaves them in good condition for a large variety of stains and is convenient and inexpensive to use. Alone, however, it does not harden tissues sufficiently to withstand the shrinking effects of absolute alcohol as used in dehydration, or the action of clearing reagents and heat, hence it cannot be used alone for objects that are to be imbedded in paraffin.

For rough work use a 10% solution of commercial formalin in water.

Tissues from vertebrate animals may be fixed in:

Formalin	10 ccm
Physiological salt solution	90 ccm

Invertebrate animals and tissues therefrom may be fixed in:

Formalin	10 ccm
Water	90 ccm
Glacial acetic acid	1 ccm

If the student proposes to do any work in plant cytology, such as the preparation of material for study of mitosis, the following fixative of Karpenchenko is excellent. It penetrates rapidly, producing a delicate and beautiful fixation of mitotic figures in root tips, ovaries and anthers.

KARPENCHENKO'S FLUID

Chromic acid	0.5 g
Water	54.5 ccm
Glacial acetic acid	5.0 ccm
Formalin	40.0 ccm

Keep a stock solution of 1% chromic acid in water and add to measured quantities of the other reagents for use.

Mercuric chloride is a powerful and rapid fixative for a large number of tissues, including invertebrate forms that are to be mounted entire, such as many aquatic organisms, some Infusoria, Cestoda and flat worms. One of the most useful of the many mixtures that have been proposed is:

GILSON'S FLUID

Mercuric chloride	5 g
Nitric acid (80% sol.)	4 ccm
Glacial acetic acid	1 ccm
Alcohol (70%)	25 ccm
Distilled water	220 ccm

Several precautions must be observed when working with solutions of mercuric chloride.

1. Never use anything but distilled water for solutions, except when working with marine organisms, when sea water is used.

2. Never bring metallic instruments into contact with the solution. Use glass or wood rods for handling the materials.

3. Materials should be left only as long as is required for complete penetration of the fixing fluid.

4. Every trace of mercury must be removed from the material before it is mounted, or small, needle-shaped crystals will eventually form and ruin the mount. Materials fixed in aqueous solutions of mercuric chloride should be washed in running water for six to twelve hours, then passed up the ascending alcohol series to 80% alcohol. Materials fixed in Gilson's fluid or any other alcoholic solution of the salt should be washed in several changes of 50%–70% alcohol, then transferred to 80% alcohol. The residual mercury must always be removed by treating the material with iodine, which combines chemically with the mercury. Add a concentrated solution of iodine to 90% alcohol by dropping until the solution is a deep amber color. After a lapse of time, depending upon the amount of mercury present, this color will be discharged. More iodine must be added as this takes place. When the reaction is nearly complete, as indicated by a slowing down of the rate of decolorization, the iodine must be added very cautiously to avoid an excess that would stain the material. If enough mercury is present to necessitate the addition of iodine after the third addition, change the entire solution to prevent precipitation of mercuric iodide on the material.

For general histological work Zenker's fluid is one of the very best. It preserves nuclei, cytoplasm and connective tissue in excellent condition.

ZENKER'S FLUID

Mercuric chloride	5	g
Potassium bichromate	2.5	g
Distilled water	100	ccm
Glacial acetic acid	5	ccm

Keep a stock solution containing the mercuric chloride and the potassium bichromate and add the acetic acid to measured quantities *immediately before use.*

WORCESTER'S FLUID

(Modification according to Galigher)

Mercuric chloride, sat. aqueous solution	100	ccm
Formalin	5	ccm
Glacial acetic acid	5	ccm

Mix immediately before use.

The original formula as proposed by Worcester contained more formalin and less acetic acid than the modification. It was recommended for fixing all forms of Protozoa. It gives splendid results with amoebae and many flagellates, such as Euglena. It does not produce as fine a fixation on Paramecia and other Infusoria as Kleinenberg's fluid, which should be used for all forms with a relatively soft cuticle.

The fixing agent should be poured upon the expanded material contained in a small amount of water. Allow to remain in the fixative at least an hour. Decant the fluid, wash in several changes of water and pass up the alcohol series to 80% alcohol. Final removal of the mercuric chloride must be effected with iodine.

Picric acid is an excellent fixative for many types of material, preserving structures with great fidelity. It does not, however, render nuclei in the best condition for staining, so a small part of acetic acid is added to the saturated aqueous solution of picric acid to improve its nuclei-fixing property. Another shortcoming is that it does not harden tissues sufficiently to withstand the macerating action of water or weak solutions of alcohol, to which end formalin is added to the mixture. Picric acid leaves the material in excellent condition for absorbing most stains, if proper precautions are taken to remove the picric acid by the methods given below.

Water and weak alcohol exert a strong macerating action on picric fixed material, for which reason all materials fixed in picric acid mixtures are transferred directly from the fixative to 70% alcohol. Never use water or alcohol of less than 50% strength for washing picric-fixed material. Wash until every last trace of the yellow color imparted by the acid has been removed. In practice, pour off the excess fixing fluid, leaving just enough to cover the material, and then add an equal amount of 70% alcohol. After a few hours have elapsed this solution may be poured off and pure 70% alcohol added. Change the alcohol frequently and do not stop the washing until every trace of acid is removed, as indicated by the absence of any color in the alcohol. Warming the alcohol to 35°C. accelerates the removal of the picric acid, but the temperature should go no higher.

The picric acid fixative given below is probably the most generally useful medium to use, since it gives satisfactory results with more materials than any other formula now in use. Nearly all stains may be used with entire success, and it is specially useful for haematoxylin

and eosin staining. Carmines work very well with materials fixed in it. Its action is so delicate that it fixes such structures as cilia and the achromatic elements of mitotic figures faithfully, making it a valuable fixative for the study of cytology. The majority of embryological, histological, botanical and zoological materials to be stained for general study are most safely handled in this fixative.

Aquatic organisms to be mounted entire, such as amoebae, Hydra and hydroids, annelids, soft Mollusca, tissues of Insecta and Crustacea, embryos of fishes, birds, mammals and reptiles, and most adult vertebrate tissues may be successfully fixed by this reagent. Due to the free acid content it must be avoided where calcareous structures are to be preserved, and it is unsuited to the fixation of flagellate and ciliate Protozoa, sponges, Medusa, flatworms and insects or Crustacea that are to be mounted whole.

BOUIN'S FLUID

Picric acid, saturated aqueous sol.	75 ccm
Formalin	25 ccm
Glacial acetic acid	5 ccm

Materials are fixed quickly, but are unaffected by prolonged immersion, although delicate structures are best removed as soon after complete fixation as possible. Contractile forms of Protozoa are best killed first in a solution of Bouin's diluted three to one with water. When they have settled to the bottom, the dilute solution may be replaced with full strength reagent. Twenty-four hours should be sufficient for thoroughly fixing most materials, and many specimens are thoroughly hardened in much less time. The material should be transferred directly

from the fixing agent to 70% alcohol, *never to water.* In this connection see the instructions given earlier for the handling of picric materials.

When very impermeable objects such as insects and Crustacea are to be sectioned, or similar refractory forms are to be dealt with, a modification of the above formula is used by substituting alcohol for the water as a solvent of the picric acid, thus:

ALCOHOLIC BOUIN'S FLUID

Picric acid, saturated sol. in 70% alcohol	75 ccm
Formalin	20 ccm
Glacial acetic acid	5 ccm

Material fixed in this solution should be handled in the same way as detailed above.

The next mixture is valuable for the fixation of many delicate structures which would be distorted by other reagents. It penetrates well and causes a minimum of shrinking. It is very useful for Protozoa with a thin cuticle, such as Paramecia, but is not recommended for Rhizopoda or Flagellata, like Euglena. Protozoa are fixed in a few minutes but may be left for several hours without harm. Naturally the time for complete hardening increases with the density and permeability of the material.

KLEINENBERG'S FLUID

Conc. sulphuric acid	2 ccm
Distilled water	100 ccm

Saturate this solution with picric acid.

Handle material fixed in Kleinenberg's in the same way as all other picric acid fixed material, by washing in alcohol, *never in water.*

Chapter IV

Dissociation

The critical examination of many forms of animal and plant tissues under the microscope may be greatly facilitated if the structures are isolated. This permits examination from every angle without interference from other structures and allows the use of selective stains on individual cells and structures to delineate their various features more clearly. The exact methods to be used in each case will vary with the nature of the material, the nature of the connective tissue and the structures. Three general methods of dissociation are recognized, each one of which performs a specific function. They are teasing, maceration and corrosion. General details of each method will be given here, with enough data for the student to select the method or combination of methods which will best dissociate the material in hand.

The simplest method of dissociating elements for microscopic study is teasing. By this method fibrous tissues not firmly united by intercellular cement may be pulled apart without previous treatment. Nerve and tendon tissues are examples of this class of material.

The actual work is performed with dissecting needles as described in Chapter XI. Two sets of needles are used, one pair of rather heavy needles with blunt points, one pair with extremely fine points to be used in the final separation of individual cells or fibers. Examine the needles carefully before starting work to be sure they are perfectly clean and sharp. If they are not sharp grind them to a fine point on a carborundum hone. When the

work is finished, or whenever work is discontinued for any length of time, clean the needles thoroughly, then they will always be in condition to resume work.

TEASING — On the stage of the dissecting microscope place a slide containing a small bit of tissue in a drop of the medium from which it was taken. With one of the stout needles in the left hand hold the material down firmly, far enough from the edge to afford a secure hold. With the other needle pull the material apart, starting at the edge and working in toward the center as the marginal structures are separated. Be careful not to crush or break the structures and avoid pressing it down on the slide. The proper technique is a pulling apart and not a crushing action. Properly done, the material is divided into fine shreds. The stout needles are now laid aside and work is continued with the fine ones. Separate the shreds carefully to isolate the individual fibers or cells of which the material is composed. When this seems to have been accomplished, transfer the slide to the compound microscope and examine under a medium power. If the individual units are not sufficiently separated, continue the work with fine needles under the compound microscope until you are sure that further division is impossible. Remove any large pieces of material that remain undivided and transfer the dissociated material to the proper reagent for mounting.

Temporary mounts of teased material may be prepared for quick study by teasing the material in physiological salt solution. It may then be studied unstained or it may be stained by applying a cover glass to the preparation and drawing the stain under the glass. The procedure is easy. Simply place a drop of stain at one edge of the cover glass and apply a piece of filter paper to the

opposite edge. The filter paper will absorb the fluid under the glass and as this recedes toward the filter, capillary attraction will draw the stain underneath. When the entire cover glass area is filled with stain remove the paper and allow the material to absorb the stain. Then repeat the same operation with distilled water and wash away all superfluous stain. 70% alcohol may be used in the same way to remove refractory stain residues. If permanent stains are used the mount may be preserved by drawing a drop of glycerine under the cover and sealing with a ring of gold size. Liquid mounts of any kind are rather unsatisfactory for a number of reasons and so are not recommended. It is better to make permanent mounts in Canada balsam as follows.

This practice is by far the best way of treating teased material of any kind for permanent mounts. By selection of appropriate stains the process may be adapted to animal as well as vegetable material, making it universal.

First fix the material in 10% formalin for at least twelve hours. Delicate structures should be pinned to thin pieces of wood, but must not be stretched.

Staining precedes teasing in this process, and is best effected by using haematoxylin as a nuclear stain and eosin as a counter stain. (For formulas covering stains see Chapter VI.) First wash the formalin-fixed material in several changes of distilled water, or for several hours in running water. When completely free of formalin, cut the material into coarse pieces and place in several times its volume of dilute alum-haematoxylin stain. After several hours' immersion pour off the stain and wash in a few changes of distilled water. Tease apart a small piece and examine it under the microscope. If the nuclei have absorbed enough stain to give them a strong, sharply

defined color, with other structures stained only slightly or not at all, wash the entire lot of material in running water for a few hours. While overstaining is not very likely, it may occur, and the material will then need to be destained by immersing it in a half-saturated solution of ammonium-alum until examination shows that all parts except the nuclei have been destained. Now wash in running water for several hours. Dehydrate by passing the material through the ascending series of alcohols. Add to the 80%, 95% and first absolute alcohol baths enough eosin to color the solution a deep pink. Leave for an hour or two in the first absolute alcohol, then pour this away and replace with new absolute alcohol without eosin and leave for an hour longer.

The material is now ready for clearing. Replace the last absolute alcohol with 50–50 alcohol and creosote-xylol as described under clearing insects and leave for one hour. Pour off this solution and add creosote-xylol, replacing this after a few hours with fresh solution. Then add balsam to the creosote-xylol containing the material, adding balsam every few hours until the liquid assumes the consistency of a medium syrup. The prepared material is then teased apart in balsam on a slide. When sufficiently isolated structures are secured remove any large pieces of tissue that may remain, add a drop of thick balsam and a cover glass. When setting the cover be sure to lower it very slowly to exclude air bubbles and to prevent the material from spreading and running to the edge of the cover.

MACERATION — Many tissues are firmly cemented together with an intercellular cement substance which would defeat any success in simple teasing. Such materials must first be subjected to the action of a reagent

which will dissolve or soften the cement and make dissociation of cell units possible by teasing or shaking.

While a great many macerating agents, as such liquids are called, have been proposed and recommended from time to time, there are only a few that need be considered. These give excellent results with most of the material the student is likely to encounter. The first is pure water, either cold or warm. Most substances, especially vegetable substances, may be satisfactorily macerated in water. Heating may be resorted to for quicker action, but the temperature must not exceed 50° C. (120° F.). Substances containing albumen should be macerated in cold water only, for heat coagulates the albumen and renders it hard.

Choice of a macerating agent is governed by the character of the material to be treated. A reagent must be selected which will exert a minimum of alteration in the tissues, yet it must perform its work thoroughly in order to separate the units completely. This is admirably accomplished by Ranvier's One-third Alcohol solution:

Alcohol (95%)	one part
Water	two parts

This is especially recommended for epithelia, glands and smooth muscle cells. Its slow action preserves tissues with the least alteration, yet so thoroughly macerates it that epithelial tissue may be shaken apart after 36 hours immersion.

Another very excellent reagent for animal tissues is Gage's Formalin Solution, made by mixing:

Formalin	2 ccm
Physiological salt solution	1000 ccm

Vegetable tissues of many kinds may be satisfactorily macerated in pure water or water and glycerin in the proportion of one part glycerin to six parts water. Objects must be left in the solution until thoroughly rotted, when the elements may be teased apart. The constituent cells of plant stems may be separated nicely by simple water maceration. To procure annular vessels for examination take the stem of maize and cut into pieces about one-half inch long. Then slice these longitudinally. Macerate in water until rotten. Place under the dissecting microscope and pick out the annular vessels.

Some difficulty may be experienced in procuring material which is quite free of debris. In such cases use a fine-pointed water color brush to pick out the desired material. The grade of brush known as a No. oo red sable is the best to use. The hairs in this brush are springy, hold their shape well and can be turned to a fine point. If the amount of desired material greatly exceeds the amount of debris present it may be better to eliminate the latter.

Sometimes the difference in weight between wanted and unwanted material may be used to separate the two groups. Repeated washings with periods of settling in between will frequently clean the material very well. Or, if a decided difference in specific gravity exists, one of the two groups will remain suspended or sink more slowly than the other group. In such cases allow most of the heavy material to settle to the bottom and draw off the supernatent liquid with a dipping tube. This operation may need to be repeated several times before perfectly clean cells are secured, but one is amply repaid for the work involved by greater clarity and beauty of the completed mounts.

Scalariform vessels and spiral vessels from vegetable stems may be isolated in the same manner as was described for annular vessels. The former may be procured in abundance from the rhizome of the fern *Pteris aquilinia,* and the stem of rhubarb (*Rheum officinale*) affords splendid examples of the latter.

To mount animal material which has been macerated and teased in water, coat a perfectly clean slide with Mayer's albumin fixative, made as follows:

Whip the whites of several eggs slightly, two or three dozen strokes being sufficient. Allow to stand one hour, skim off the foam and pour the remaining liquid into a clean graduate. Add an equal volume of glycerin and one gram of sodium salicylate for each 100 ccm. of liquid. Shake thoroughly and filter through paper into a clean bottle. Filtration is very slow. If a vacuum filter is available it should be used. Lacking this, the operation may be accelerated by placing only a small portion of solution in the filter at a time and replacing the filter paper frequently. Keep a working stock in a small vial fitted with a dropping rod, and the reserve stock in a tightly closed bottle.

The slide is prepared by placing a drop of the fixative on it and smearing it back and forth with the clean finger, rubbing it quite vigorously to make sure it adheres to every portion of the slide. Wipe off the surplus with the moist finger until only a very thin film remains. For a method of cleaning slides see Chapter V.

Place a drop of water containing the desired material upon the albumenized slide and tilt it back and forth a few times to spread the material. When almost dry, dip the slide in 95% alcohol to coagulate the albumen and hold the material fast to the slide. Pass the slide down

through the descending series of alcohols to water, then to haematoxylin stain, if this is to be used. Leave until the nuclei are stained deeply. Wash in running water for fifteen or twenty minutes and then pass up the ascending alcohol series to 95% alcohol. Counter stain for two minutes in 0.5% eosin in 95% alcohol. The depth of the eosin stain may be reduced in 95% alcohol if too deep. Place the slide in creosote-xylol for several minutes, or until clear, transfer to pure xylol for a few minutes, then add a drop of balsam and a cover glass.

If stains other than haematoxylin are to be used, the technique should be altered to coincide with that required for the particular stain selected. Consult Chapter VI for methods.

Corrosion — The dissociation method known as corrosion makes use of the power of certain chemicals to destroy some structures without affecting others. This process makes it possible to study such structures as fish scales, sponge spicules, and chitinous insect skeletons. Another application of corrosion consists in injecting the internal cavities of organs, insects, shells, etc. with wax, then corroding away the soft parts with a strong reagent, leaving perfect casts of the interior surfaces.

Since calcium is soluble in acids, calcareous structures such as some sponge spicules, Foraminifera, fish scales and the like, must be freed of soft tissues by maceration in strong alkali solutions. Concentrations of 30–35% of sodium or potassium hydroxide in water are the usual strength. At room temperature several days to as many weeks may be required to completely dissolve the soft tissue. The action of the reagents may be accelerated by boiling. While this is harmless to calcareous structures it must not be attempted with insects, for it would destroy

them. When all unwanted parts have been dissolved, wash the material thoroughly in water to cleanse it of any alkali, dehydrate and mount in balsam.

Siliceous structures such as radiolarians, diatoms and the spicules of siliceous sponges are cleaned with strong nitric acid. The material is immersed in the cold acid and left to dissociate, or it may be boiled for more rapid results. Wash, dry and mount the cleaned specimens in balsam.

Hard materials such as bone, teeth, horn, claws, coal and minerals require different treatment, to be described in a succeeding chapter.

Chapter V

Section Cutting

In order to complete the detailed microscopic study of organisms and tissues with the various parts in their correct relative positions, it is necessary to cut sections thin enough to transmit light. This must be accomplished without distorting the specimen or crushing any of its parts. Some vegetable materials such as soft stems, roots, fleshy leaves, etc. may be sectioned free-hand after a little practice, but animal tissues must be properly supported in a plastic medium to hold the delicate parts in their proper positions.

If work with a large variety of tissues is contemplated a mechanical device called a microtome is necessary. This instrument holds the material to be cut and passes it over an extremely sharp knife which shaves off thin sections. It is automatic in action and very accurate, advancing the specimen at predetermined intervals between each cut, so that any number of uniform sections may be made. This instrument, however, is expensive and is beyond the reach of the average student. To meet the demand for an inexpensive device to serve the same purpose the hand microtome or well microtome was contrived. This permits the cutting of sections to fulfill every requirement of the student. A home-made well microtome is illustrated in Chapter XI. Anyone with the necessary facilities can build such a microtome, or have it made in a machine shop at small cost.

As each section is cut from the specimen, it is placed on a piece of clean paper, keeping the sections in their cor-

rect consecutive order. In this way the entire morphology of a specimen may be reconstructed. The sections may be placed serially on the slide and a progressive study of the entire organism effected.

Animal tissues must be supported in some medium when they are to be sectioned. Three general methods are in use, namely, paraffin imbedding, celloidin imbedding and freezing. When materials are to be used for diagnostic purposes or for immediate examination sections may be obtained by freezing. Celloidin (nitrocel-

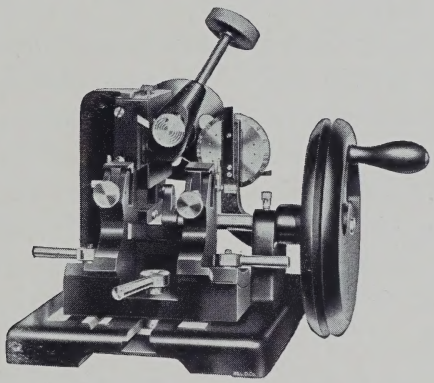


Fig. 30. Bausch & Lomb Minot Rotary Automatic Microtome for laboratory use in paraffin sectioning.

lulose) is used for sectioning a few materials, such as whole brains or some watery tissues which would shrink in hot paraffin. Aside from these exceptions, paraffin is used for supporting practically every material the student is likely to encounter, except calcareous tissues and minerals. It is the most valuable method of microtechnique and is essential to practically every field of biological science. A complete understanding of this operation as well as considerable skill in its execution should form part of the equipment of every student.

After the material has been properly fixed and washed, it must then be dehydrated. This is accomplished by replacing the water with purified methyl alcohol. Alcohol has the property of shrinking tissues and distorting the cells when the material is placed directly into a strong solution. For this reason the alcohol is diffused gradually by using dilute solutions. Dehydration must be gradual and it must be thorough. If the least trace of water remains in the tissue it will prevent proper penetration of the clearer and the paraffin, thereby rendering the whole operation a failure.

The alcohol series usually starts with a 15% solution of 95% methyl alcohol which need not be absolutely precise. For practical purposes the solution may be prepared by adding 15 ccm. of 95% alcohol to 85 ccm. of distilled water. All other percentages may be made in the same way; 35% solution is 35 ccm. alcohol to 65 ccm. water, 50% solution is 50 ccm. alcohol to 50 ccm. water, 70% solution is 70 ccm. alcohol to 30 ccm. water, etc. Steps of 15%, 35%, 70%, 80%, 95% and 100% or absolute alcohol are generally used for all but the most delicate material.

Wide-mouthed bottles with well-fitting stoppers are the proper containers. It must be possible to transfer the material quickly from one jar to the next, so containers with even moderately narrow necks should be avoided. Mayonnaise jars in the eight-ounce size are excellent.

If the material has been washed in water it is placed first in the lowest member of the alcohol series. If it has been washed or preserved in alcohol, such as material fixed in any of the picric acid mixtures, dehydration has started and should be continued from that point by transferring the material to the grade of alcohol next higher to

that used for washing or preserving. Successive changes to higher concentrations are then made until absolute alcohol is reached. The volume of alcohol must at all times be several times that of the material.

The student may experience some difficulty in handling small objects when transferring them from one reagent or solution to the next. Large objects are easily handled by pouring off one grade of alcohol, and adding the next. This is difficult with small, light specimens such as mosquito larva, plant lice, aquatic organisms and the like, so these are transferred on a filter.

Cut a three-inch disc of filter paper from a larger sheet, and place it in a small funnel measuring one and one-half inches across. Pour the material to be dehydrated, with its containing liquid, into the filter. If the liquid is to be retained allow it to filter into an appropriate container. If of no value discard it. The next solution, say 15% alcohol, is now poured into the filter and allowed to drain into the container. Now remove the filter from the funnel and place it in the container of solution, where it is allowed to remain until the time comes to transfer it to the next higher step, when the filter is drained, replaced in the funnel and the appropriate solution allowed to drain through it and again placed in the container. This method, while somewhat wasteful of alcohol, permits the handling of large groups of small objects without loss.

Another method of drawing off solution is by means of a pipette. In this method test tubes or vials may be used as containers, cutting down the amount of alcohol needed. An ordinary straight glass tube or a regular pipette serves the purpose. The disadvantage of this method is that small objects are easily drawn into the pipette and thus lost. To avoid this possibility the author ties a piece of

filter paper over the immersion end of the pipette. First a small wad of cotton is stuck loosely into the pipette end, leaving a small ball projecting from the tube. The filter paper is then folded loosely over the end and secured with thread. If cotton is not used the pipette end is apt to puncture the paper, thus defeating its purpose. If diffusion through the filter paper is slow it may be speeded up by gentle suction applied to the free end of the pipette.

The period of immersion in the various alcohol steps cannot be stated accurately. Small porous objects may be completely permeated in less than an hour, while dense tissues, such as liver, will require several hours for the liquid to penetrate to the center. It is not necessary for material to remain in each step until completely penetrated, since the weak alcohol in the outer layers will penetrate to the interior while the stronger grade is entering the outer layers. As a matter of fact no material should remain longer than six hours in an alcohol solution of less than 50% as the macerating effect of a weak solution will disintegrate the tissue. The time in alcohols of 70%, 80% and 95% should be sufficient to insure complete penetration, and may safely be extended to days.

Change the absolute alcohol at least once and preferably twice, and be sure to leave the objects until completely dehydrated. A good rule is to leave it until you are sure every trace of water has been removed, then leave it as long again.

The dehydrated material is now in condition to absorb a reagent in which paraffin is soluble. This process is called clearing because the oils or other reagents which dissolve paraffin also tend to clear the objects and render them somewhat transparent.

Many clearing agents have been tested, but experience

shows that toluol, a by-product of coal-tar, is the best. It penetrates rapidly, does not harden tissues too much and is so thin and volatile that it is quickly removed from the paraffin. Many writers on microtechnique recommend xylol for clearing, but this chemical frequently renders tissues as hard as stone, making cutting difficult or impossible. The student is well advised, therefore, if he uses toluol for clearing in all paraffin work.

Mixtures of alcohol and toluol must be stored in tightly covered containers as they quickly absorb water from the air. For the same reason material must be handled as rapidly as possible when changing from one solution to the next.

The old practice of taking material out of a reagent of one specific gravity and placing it immediately into one of another gravity accounts for many poorly prepared microscope slides. The transfer from absolute alcohol to toluol is a case in point. Clearing, like dehydration, must be done gradually if the material is to remain in the best condition. The steps need not be as numerous as those recommended for dehydration, except in the case of extremely delicate material, three steps being sufficient for student material.

After removing the material from the last absolute alcohol bath, place it in a mixture of one part toluol to three parts absolute alcohol. Experience is the only guide to the length of time material must remain in each bath. As a general rule allow it to remain in each alcohol-toluol mixture as long as it remained in each of the higher grades of dehydrating alcohol. Next transfer the material to a mixture of equal parts of alcohol and toluol, and, after the necessary immersion time, transfer it to pure toluol.

Properly cleared material will present the same appear-

ance throughout. If dark spots are seen when it is held against a light or if these spots appear white and opaque when the specimen is held in a bright light against a dark background, they are an indication that the clearing is not complete and the material must be returned to pure toluol, preferably a fresh batch, for further clearing. If the spots still persist after prolonged immersion in the clearer, the trouble is caused by incomplete dehydration. This can be rectified only by replacement in absolute alcohol for a longer period and again clearing in three stages.

Over-immersion in toluol does the material no harm, so leave it until there is no question of incomplete penetration. Then replace the used toluol with a fresh solution and leave it for another period of time to insure complete removal of every trace of alcohol. Unless penetration of the toluol to the very center of the material is complete it cannot be infiltrated fully with paraffin.

Two grades of paraffin are used for sectioning. One, soft paraffin, is suitable for imbedding materials to be cut into rather thick sections (14 μ or over) . This has a melting point of 45° C. (115° F.) . Hard paraffin melts at 54° C. (130° F.) and is used when thin sections (12 μ or less) are to be made.

Commercial paraffin varies considerably in quality. Each lot must be tested for melting point and homogeneity before it is used. First test the melting point, which will probably be low. Then fill a small pill box with melted paraffin and cool it as quickly as possible in cold water. Examine it critically and if it shows white, opaque spots it is unfit for use.

When a satisfactory lot of paraffin is found it must be filtered to remove foreign substances. A heated funnel

is the best way to filter paraffin. If a small quantity of wax is heated well above its melting point it may be filtered through a previously heated metal funnel before it solidifies. The filtered paraffin may be collected in containers which have been smeared with a thin coat of glycerin to prevent the wax adhering to them.

The grade of paraffin known as Parawax is available in all grocery stores, and serves quite well as a soft paraffin. The author has used it for hand-sectioning with the well microtome with very gratifying results. Each lot must be tested for melting point and filtered before use. This product varies considerably in melting point so that by testing several lots one may be found with a melting point high enough to make it available as a hard paraffin.

The melting point of paraffin may be raised to produce a hard paraffin by adding bleached beeswax to it. No exact proportions can be given because of wide variations. It is advisable to melt Parawax and then add beeswax a little at a time. Take samples frequently and chill them in cold water to solidify. When the samples are hard, test for melting point. Samples of Parawax and beeswax have been found which when combined produced a hard paraffin melting at 60° C. (140° F.), with good cutting quality. When a suitable product has been secured guard it carefully, for it is a rare treasure.

Another method of raising the melting point of paraffin is given by Lee in his Vade-Mecum. "Paraffin of about 50° C. melting point is taken and heated in a porcelain capsule (evaporating dish or crucible) by means of a spirit lamp. After a time disagreeable white vapors are given off and the mass shrinks a little. This result is arrived at in from one to six hours, according to the quality of the paraffin. The mass then becomes brownish-yellow,

and after cooling shows an unctuous or soapy surface on being cut. The melting point will be found to have risen several degrees."

Save all scraps of paraffin, such as unused portions of blocks, shavings, chips and trimmings, for reheating tends to improve the cutting quality. When a quantity has been collected melt it, filter and determine its melting point.

The material is now in pure toluol and ready to be infiltrated with paraffin. Gradually saturate the toluol with paraffin at room temperature by adding finely shaved paraffin to it. If the material is very fragile the paraffin must be supported while passing into solution. This is done by tying the paraffin up in a cheesecloth bag and suspending it in the toluol by a string so that the paraffin dips below the surface of the toluol but does not touch the material. As the paraffin dissolves, add more until the liquid is saturated.

Material may be left in this mixture for days without suffering any ill effects, and should be left for at least twenty-four hours before going on to the next step. Now place the container in a warm place where it will receive very gentle heat. If a paraffin oven is available it may be set on top of this, or it may be placed on a water bath or sand bath that is held at a temperature lower than the melting point of the paraffin. Add paraffin until no more will dissolve. Allow to stand over night with the container uncovered to permit evaporation of the toluol.

Now place the container in the paraffin oven and allow the paraffin to melt completely, gently agitating the dish occasionally to thoroughly mix the contents. The oven should maintain a fairly constant temperature in the neighborhood of 54–55° C. (130° F.) for either hard or

soft paraffin. (For description of a paraffin oven see Chapter XI.)

Penetration of the paraffin to the interior of the specimens will depend upon their size and permeability. Large pieces of fairly dense material may remain two hours, small pieces or less dense tissues one hour, and small, easily permeable material for twenty to thirty minutes. At the end of this period pour off half of the paraffin-toluol mixture and make up to original volume with pure melted paraffin, agitating the container to mix thoroughly. At the end of the respective periods pour off all of this mixture and replace with pure melted paraffin. Change the pure paraffin several times while the material is in the oven to insure absolute elimination of toluol.

The period of immersion in pure paraffin is again dependent upon the material. Small, easily-permeable objects will require about two hours, large pieces of dense material will require more time. Six to eight hours should suffice to infiltrate even the most refractory substances, if the preliminary infiltration has been thorough. Prolonged immersion extending several hours beyond the actual time needed will do no harm if the oven is at the proper temperature. High temperatures are injurious, and material which has been cooked is worthless.

The infiltrated material is now ready to be imbedded for sectioning. The objects are taken from the paraffin bath, placed in a shallow container with enough melted paraffin to cover them and arranged in the desired positions for cutting. Two requirements must be met in imbedding. First, the individual units of material must be so arranged in the paraffin cake that they may be cut apart and sectioned in the desired plane. Secondly, the paraf-

fin must be cooled as quickly as possible to prevent crystallization.

Choice of an imbedding container may be made from several available objects. For small objects there are watch glasses, which may be had in a number of sizes from laboratory supply houses. Paper trays made as shown in Fig. 31 are inexpensive and very efficient. Select a heavy bond paper or light cardboard and fold as indicated. The tray has the advantage that any required data may be written directly upon it, dispensing with separate labels.

The operation of imbedding should be done directly in front of the paraffin oven, or close to it. A small spirit

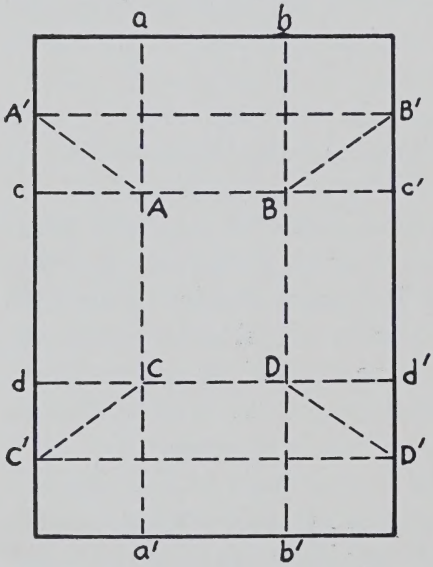


Fig. 31. Diagram of paper imbedding tray. Fold along lines a-a′ and b-b′. Without unfolding make the folds A′–B′, c-c′, d-d′ and C′–D′. Indent and fold along A′–A, B′–B, C′–C and D′–D. Unfold and refold so the area A–B–D–C forms the bottom of the tray. Fold the ends down on the outside.

lamp, two fine dissecting needles, fine-pointed curved forceps, imbedding containers and a dish of cold water should be at hand.

When all is ready smear the inside of the imbedding container very lightly with glycerin. Only the thinnest

film is necessary and all surplus should be wiped away. Pour into it enough paraffin to cover the material thinly. Now, with warmed forceps quickly transfer the objects to the dish and arrange them properly. If several objects are being imbedded arrange individual pieces in an orderly progression, allowing several millimeters of paraffin between each. This is to make it possible to separate one unit from the others without destroying them. It is usual to place objects so that the intended plane of section is parallel with the bottom of the dish. This is obviously impossible with some material, for long, thin specimens cannot be stood on end. Handle objects in melted paraffin with the greatest of care as they are very apt to crumble or break.

Should the material consist of very small or fragile objects which cannot be individually handled, pour off most of the melted paraffin, then quickly pour the remainder containing the objects into a prepared dish. Arrange them with warmed needles. Should the paraffin begin to solidify before arrangement is complete it may be kept fluid by warming the dish just a little by placing it on top of the oven or on a warmed metal plate. Be extremely careful not to get it too hot. If a film of semi-solid paraffin forms on the surface warm a section lifter and pass it lightly over the surface. The upper layers of paraffin may be kept fluid for a few minutes in this way. Heating of paraffin with imbedded objects is recommended only as a last resort, for the objects sink to the bottom of the dish, leaving their under sides unsupported, and the heated dish retards cooling enough to permit some crystallization of the paraffin.

Now take the dish in the fingers and move it carefully to the dish of cold water where it is held almost sub-

merged. Blow on it gently to hasten cooling of the top layers of paraffin. As soon as a thick film has formed on the surface of the paraffin lower it slowly in the water and hold it completely submerged until it solidifies. This must not be done too rapidly or the rush of water over the rim will cause the center to spurt upwards and ruin the preparation. If a paper tray is used for imbedding it is only necessary to float the tray on the surface of the cooling water.

If the tap water is warm it is advisable to add ice to the chilling water, for the object is to cool the paraffin as quickly as possible to prevent crystallization. When the cake is completely cool it should float free of the imbedding dish and rise to the surface of the water. If this does not take place in half an hour run a thin blade around the edge of the cake to loosen it. Never heat a dish to release the block of paraffin.

Examine the paraffin blocks carefully. They should present a homogeneous appearance, without white spots or bubbles. If opaque areas occur around the imbedded objects it is an indication that all the clearing agent was not removed. The only remedy for this condition is to put the blocks back into the oven, melt the paraffin and keep it fluid for several hours to evaporate the clearer, changing the paraffin once or twice, and then reimbed the material. Objects thus imbedded may be sectioned at once or kept in a cool place protected from dust.

When the time comes to cut sections the paraffin cake must be separated so that each small block contains only one object. If the objects are very small and close together several may be included in one block. To cut objects apart make a scratch on the surface of the cake to indicate the intended cut, then gradually deepen this un-

til the cake may be broken apart. Trim the edges square, leaving several mm. of paraffin surrounding the object on all sides. Be sure the intended plane of section is correct and then true up the edges so that a true rectangle will be presented to the knife.

If the sections are to be cut free-hand, that is, without a microtome, it is advisable to fasten the paraffin block to a support to afford an easy grip in the hand. Such a support is provided by a small block of wood, about three-

Fig. 32. Cutting sections free-hand with a razor.

quarters of an inch square by three inches long. Dip the end of this into melted paraffin to coat it to a depth of about one-eighth of an inch. This stratum of paraffin is used to fasten the paraffin block to the support and should be left permanently. If the student has access to a well microtome or an automatic microtome the following instructions will apply with equal effect.

Place the paraffin block with the imbedded material on the table, with the face to be cut on top. Warm a flat

metal instrument such as a scalpel handle and apply it to the paraffin on the support block until this is slightly melted. Remove the warm instrument and quickly press the paraffin block into the melted paraffin. Now heat a needle or the scalpel handle again and melt the paraffin at the joint between the two blocks, welding them together solidly. Immerse the whole in water for several minutes, when it will be ready for cutting. Trim the object block to a perfect rectangle before beginning to cut sections.

Where many sections are to be cut an automatic microtome is almost necessary, but for the student who makes only a few sections at infrequent intervals it is not essential. Section cutting free-hand is not difficult and after a few trials very thin sections may be produced. Fig. 32 shows the method of holding the knife and the object for free-hand sectioning. The well microtome, shown in use in Fig. 33, is an improvement over free-hand cutting. This instrument is excellent for student use, since it is inexpensive and reliable. By fastening the object block to the support block in the microtome the object is held securely while the large table provides a solid, steady surface on which the knife slides. Adjustment for section thickness is secured by turning the screw at the bottom of the well.

One item of great importance in successful sectioning is the knife used. This must be stiff and very sharp. A safety razor blade may be used if it is fastened to a handle to stiffen the blade and hold it rigid. Blades of the single-edge type backed with metal heavier than the blade are stiff enough for sectioning, but the double-edge blades are too flexible. Better than any safety razor blade is the old-fashioned straight razor used

by barbers. This is heavy and stiff and if of good quality will retain its edge for a long time. Give it the same care you would your shaving razor, for the edge must be just as keen for sectioning as it should be for a comfortable shave, or even sharper. Keep the razor in perfect condition by honing on a good hone, and by frequent strop-

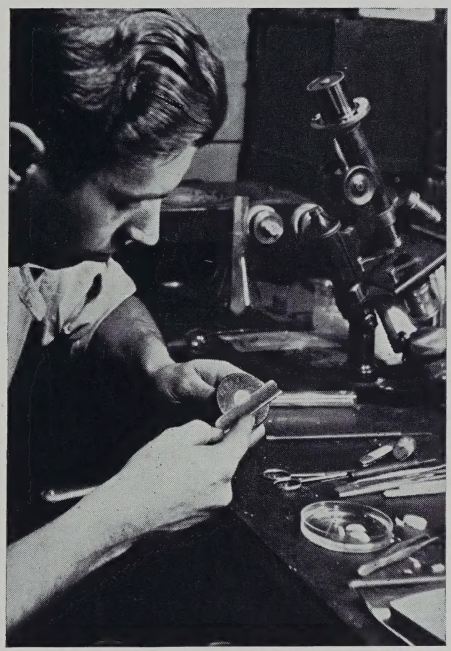


Fig. 33. Cutting sections by hand in a well microtome. Sections imbedded in paraffin.

ping. If any difficulty is experienced in keeping the razor perfectly sharp, take it to a barber and have him put it in condition. Once properly sharpened the razor may be used for cutting many sections before sharpening will again be necessary.

Several difficulties may be encountered in section cut-

ting, and it is well to know their cause and remedy. If the material or sections are split or cracked in the direction of the cut, the knife may have a nick in it or there may be hard particles in the material. Clean the knife with xylol and try again. If the scratches disappear they were caused by hard particles from the material or paraffin. They may be corrected in the same way if they reappear. If this expedient has no effect, try using another portion of the knife and note results. If the cracks disappear the knife was at fault and should be ground and honed to clear it of nicks. If the material crumbles and tears out of the paraffin this may be caused by insufficient infiltration. This is indicated by a soft, mushy consistency of the paraffin around the object. It may be caused by incomplete dehydration or insufficient time in the paraffin oven. To correct it dissolve the paraffin with xylol, dehydrate and reimbed. If the material is hard and brittle, the clearing agent may be at fault or the material may have been subjected to too high a temperature in the paraffin oven. To remedy this condition read again the paragraphs covering these two subjects.

As the sections are cut they should be placed in a row on a sheet of paper perfectly free from dust or lint. Arrange them in serial order so that a series of sections may be placed on the slide in the order which they occupied in the live object if desired. In this way the entire morphology of the specimen may be reconstructed on the slide.

Cut the sections with a straight motion of the knife. Hold the microtome in the left hand, as shown in Fig. 33, with the knife in the right hand. Pass the knife straight through the material and paraffin with a quick, steady motion. Do not see-saw the knife back and forth as this

is very likely to leave zig-zag lines on the section. Some practice will be necessary, of course, before the correct technique is mastered, but once attained, uniform results may be secured with certainty.

PREPARING THE SLIDES — The slides must be perfectly clean and free from any trace of grease. Clean the slides by boiling in the following mixture:

Potassium bichromate	20 g
Water	100 ccm
Conc. sulphuric acid (commercial)	75 ccm

Rinse the cleaned slides in water, wash with ammonia, then distilled water and store in 90% alcohol until needed. When they are removed from the alcohol for use they will dry almost instantly and be perfectly clean.

Mayer's albumen fixative is needed. Prepare a slide as directed in Chapter IV by smearing with Mayer's fixative. Place a drop of distilled water on the slide and lay the section on it in the place it is to occupy permanently.

The section will be curled somewhat and must be flattened by the application of gentle heat. Be extremely careful not to overheat as high temperatures are sure to distort the cells, even to the point of producing characteristic cracks in which cell masses are pulled apart, often in parallel lines. This distortion becomes more evident after the paraffin is removed and the sections stained.

The process of flattening sections is called stretching, and as a general guide it may be said that sections in soft paraffin should be stretched at about 37°C. (98°F.), those in hard paraffin at 40°C. (105°F.). The slides may be placed in the paraffin oven which must be adjusted to maintain the above temperature, or a warming stage may be made by filling a pan with sand and equipping it with

a thermometer. Bring the sand temperature up to the required point, cover it with a sheet of glass and lay the slides on the glass.

The length of time required to stretch sections depends upon their thickness, smoothness and the character of the material. Sections of cylindrical objects are especially hard to flatten. Some sections flatten perfectly in ten minutes at the above temperatures while others will take an hour or more. Particularly difficult subjects may require heating high above an open flame, but be very careful not to melt the paraffin. As a last resort, to be used only in the most obstinate cases, the sections may be flattened with a rolling motion of the little finger. Add distilled water as required to make up that lost by evaporation. Sections must be kept moist all the time.

When the sections are flattened, remove the slides from the warming pan and carefully pour off any excess water, leaving them just wet enough to prevent the sections from sticking. If the sections have shifted their position take a needle and carefully move them into place, touching only the paraffin and not the material itself. Now place the slides in the oven or on the warming pan for about half an hour, when they may be placed in boxes for later finishing. If they are to be finished at once they may be dried in the oven at 37°C. (98°F.) or allowed to stand twenty-four hours if not dried by artificial heat. Slides thus prepared may be stored for years in tightly covered boxes in a cool place and finished whenever needed.

Laboratory supply houses furnish special containers for the reagents used in treating paraffin sections affixed to slides. These are called Coplin jars, are made of glass and are provided with vertical slots to hold the slides. The student can get along quite well with substitutes in

the form of straight-side vials with bakelite screw caps. These are procurable from drug stores in a size one and one half inches in diameter by four inches high, just a convenient size for slides. One or several slides may be handled at the same time by making a little basket of brass wire to hold them. Thus the slides need not be touched and the work is reduced to a minimum.

Arrange the reagent jars as in the diagram (Fig. 34). In front of each row of jars place a strip of blotting paper to be used in absorbing the excess liquid that drains from the slides.

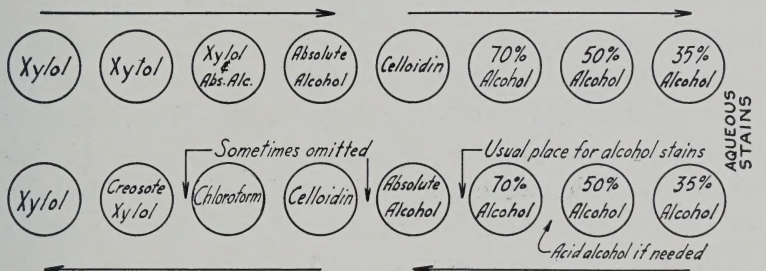


Fig. 34. Diagram showing arrangement of reagent jars for handling and staining sections fixed to slides.

Removal of Paraffin — Place the slides in the first jar at the left, labeled, "Paraffin Xylol No. 1." Leave for two to three minutes, remove and allow to drain into the jar, touch the ends to the blotting paper and quickly place in the second jar labeled, "Paraffin Xylol No. 2." They may remain in this jar for a long time without harm, although several minutes is sufficient.

On removal of the slides from the second xylol place them in 50–50 xylol-alcohol to begin removal of xylol. Two or three minutes is sufficient. Transfer them quickly to the absolute alcohol *without draining or blotting*. At this stage and on transfer to thin celloidin, great

caution must be observed to work quickly, for the slides must not at any time be allowed to dry. Both of these reagents are highly volatile.

COATING WITH CELLOIDIN — Transfer the slides quickly from absolute alcohol to a thin solution (about 1%) of celloidin in 40% ether-60% alcohol. After several minutes lift the slides and allow them to drain thoroughly. Watch them closely and when the celloidin adhering to them forms a soft film, plunge them into the jar of 70% alcohol. Lower them to the bottom of the jar with a single motion as any hesitancy will cause a ridge to form in the celloidin. Allow the slides to remain in 70% alcohol about three minutes.

If these directions have been followed carefully the sections should now be firmly fixed to the slides. If they fall off or loosen and become wrinkled it is probably because the slides were not perfectly free from grease, the sections were not perfectly flattened, or they were made too hot during the stretching operation.

TRANSFERRING TO STAIN — The sections are now ready to be stained. If an alcoholic stain is to be used they are transferred directly from 70% alcohol to the stain. If a water stain is used for nuclei the slides are passed down the series of alcohols to water as instructed in Chapter VI, which should be consulted for staining and counterstaining techniques. The student will be well advised to use alum-haematoxylin for a nuclear stain with eosin as a counterstain in his early efforts, and should master these before proceeding with alcoholic stains.

CLEARING — Properly stained, counterstained and dehydrated, the slides are now cleared in xylol to make the tissues transparent. Xylol exerts a strong shrinking action on tissues, so the sections are protected against this by

recoating with celloidin. Transfer the slides from absolute alcohol to 1% celloidin in ether-alcohol and treat exactly as described for the first celloidin coating, except that instead of hardening the celloidin in alcohol we now plunge the slides into a jar of chloroform where they may remain for a minute or two, but no longer.

Transfer the slides from chloroform directly to 50–50 creosote-xylol for final clearing. They should remain until every trace of cloudiness has disappeared. When cleared, pass them to a jar of pure xylol and leave for three to five minutes. Longer immersion is not advisable because of the shrinking and hardening effect of the xylol.

MOUNTING — Canada balsam is used almost exclusively for mounting paraffin sections. Have at hand a supply of clean cover glasses, a bottle of balsam, fine-pointed forceps, and a small spirit lamp.

Take a slide from the jar of xylol, drain thoroughly and with a soft cloth wipe the back and front of the slide clean, up to the section. Hold the slide in the left hand, place a drop of balsam on the section and with the forceps in the right hand pick up a clean cover glass. Warm this over the flame and lower it quickly into place. Properly done there should be no air bubbles, but if there are small ones they will probably work out as the slide dries. Large ones, however, must be treated by dissolving off the cover with xylol and setting a new cover.

SECTION CUTTING BY FREEZING — When sections are required in a hurry and time does not permit paraffin imbedding, satisfactory sections may be cut from pieces of frozen tissue. While not generally suited to the cutting of sections intended for critical study, the method is still of considerable value for diagnostic, identification and demonstration purposes. Fresh tissue may be frozen and

sectioned without any previous treatment although this is not the best practice. Whenever time is available the material should first be fixed in a 10% solution of formalin in water or physiological salt solution. If the pieces are cut rather thin, fixation will be complete in ten to twelve hours with most materials. Most of the routine staining techniques may be used with entire success on formalin-fixed material. If the sections have been cut from unfixed material the best practice is to place them in 10% formalin at room temperature for ten to fifteen minutes before staining. When freezing slices of material which have been fixed in formalin, wash them for several hours in running water before cutting sections. If sections are required from material preserved in alcohol, run the material down the alcohol series to water before freezing.

Fixed and washed material may be frozen and sectioned "as is," but better sections will usually result if it is infiltrated with a gum and syrup mass. This supports the various elements and freezes without the formation of ice crystals that might otherwise injure delicate tissues.

To make the infiltrating mass take:

Gum acacia (gum arabic)	40	g
Water	60	ccm
Carbolic acid crystals	0.5	g

Dissolve the gum in the water and add the carbolic acid. To this solution add 40 ccm of syrup made by saturating water at room temperature with cane sugar. *Do not heat the mixture.*

Transfer the thin slices from water to this solution and leave until thoroughly infiltrated. This may take twenty-four hours, or the material may be left for days without

harm. The prepared material is placed on the object carrier of the microtome with a few drops of water and frozen.

The usual method employed in laboratories is to freeze the material in special apparatus using liquid carbonic acid gas. The average student will not have such equipment at his disposal, so he must resort to other methods. Two alternatives are available, one in which solid carbon dioxide or dry ice is used, the other by the use of ethyl chloride. The worker will have to select the method best suited to his conditions.

Dry ice may be purchased in small quantities from large dairies or ice cream manufacturers. A cube measuring one inch square is ample for freezing several pieces of tissue. Place the dry ice in a box made of corrugated cardboard and lay the tissue directly on the ice. Then cover the box with another piece of board and leave until frozen. Cut it free from the ice and place it on the object carrier of the microtome, which has been prepared by placing a few drops of water on it. Now lay the dry ice on top of the tissue and leave for a few minutes until thoroughly frozen. Remove the ice and cut sections with a chilled knife.

CAUTION: When handling dry ice be very careful not to allow it to come into contact with the skin. Always handle it with forceps as the extremely low temperature can and does cause painful burns.

The ethyl chloride method is perhaps slightly more convenient to use, since the freezing agent is more readily available. Ethyl chloride may be procured from any druggist in 50 g and 100 g tubes, costing about two dollars for the larger size. It comes in liquid form in glass tubes fitted with a release valve which liberates the liquid.

The chemical works on the principle of lowering the temperature by rapid evaporation. It is so highly volatile and evaporates at such a rate that by this evaporation it extracts heat so rapidly that it freezes the tissue with which it comes in contact.

To use ethyl chloride for freezing, place a few drops of water on the object carrier of the microtome and freeze this by directing a stream of ethyl chloride against it from the nozzle on the tube. Quickly place the tissue on the object carrier and freeze in the same manner. When frozen cut sections in the usual way as rapidly as possible.

When sectioning any frozen material, work rapidly and keep the knife chilled with ice. Dip the knife in ice water before each cut, for unless the knife is kept cold the sections will stick to the blade. If the work is being done on a rotary or a sliding microtome, a piece of ice may be placed on the back of the knife, where by melting it will soon shape itself to the blade and remain in place. This is obviously impossible when using a well microtome, so the knife must be kept cold by dipping it in ice water.

If the tissue has been frozen too hard the sections will crack or roll. In this case wait a short time and try again. When the consistency reaches the point where perfect sections may be cut, work rapidly and cut as many sections as are needed. If the material becomes too soft before the required number of sections are cut, freeze it again and continue the work.

Handling Loose Sections — Sections cut from frozen material, or free-hand sections of hard substances, are called loose sections, as distinguished from paraffin sections affixed to slides for processing. These are sometimes handled through the operations of staining and mounting simply by transferring them from one reagent

to the next until xylol is reached, when they are mounted in balsam. Better results are secured by fastening the sections to slides by Wright's method, which permits more convenient handling and prevents loss of loose portions.

WRIGHT'S METHOD — Change the water on the sections several times to wash out all gum. Select a good section and pass a slide under it in the containing water. Lift the slide carefully so the section is carried with it, holding it in place with a small brush if necessary. See that the section lies perfectly flat on the slide. Drain off superfluous water, being careful that the section does not move, and blot off the surplus water up to the section with filter paper. With a pipette flood the section and the slide immediately around the section with absolute alcohol, but do this gently to avoid moving the section. If it should move be sure that it lies perfectly flat and is free from folds before continuing to the next step. Allow the absolute alcohol to remain a few seconds, then drain the slide and immediately place a few drops of 1% celloidin on the section. Tilt the slide several times to distribute the celloidin, then raise to a vertical position and blot off the excess solution with filter paper, leaving a thin film on the section and slide. During this manipulation be sure the section has not slipped or folded. As soon as the celloidin film begins to set, plunge the slide in 70% alcohol to harden the celloidin.

The sections are now firmly attached to the slide and may be treated as instructed for paraffin sections. All operations are identical with that schedule, except for dehydration in absolute alcohol. This reagent is a solvent of celloidin, hence would dissolve away the film that holds the sections to the slides. In place of alcohol we make use of the dehydrating property of creosote-xylol

by transferring the slides from 95% alcohol (in which they should remain no longer than five minutes) to equal parts of creosote and xylol. Change this solution after every trace of cloudiness disappears from the slide, then allow it to remain in a second change of creosote-xylol twice as long as it remained in the first solution. When clearing and dehydration are complete mount in balsam.

Chapter VI

Staining

The primary purpose of preparing materials for the microscope is to study their various elements. The greater proportion of microscopic elements are colorless or nearly so, and, in order to increase their visibility or to distinguish certain elements from others, the material is subjected to various dyes or color-bearing chemicals which impart color to parts or all of the specimen. Certain cellular and intercellular substances absorb these stains in varying degrees. Some parts will be strongly colored while others retain little or no stain, thereby affording an opportunity to differentiate sharply between various elements. Some stains are designated as nuclear stains because they possess an affinity for the nuclei of cells, coloring them deeply, while the cytoplasm is colored lightly or not at all. Other stains fall into the group known as general or plasma stains. These impart a color to the cell plasm, affording us an opportunity to stain the nucleus with one color and the plasm with a contrasting color, thus presenting details with maximum contrast.

The most widely used stain for most sections and for some whole material is haematoxylin, a dye prepared from logwood. The active principle is haematin, a complex compound of carbon, hydrogen and oxygen. When combined with salts of aluminum, iron and some other metals that act as mordants, this dye produces lakes of a deep blue or black color in the nuclei of cells, imparting to them a strong, precise, permanent color.

Haematoxylin alone has almost no staining power but when dissolved in water it oxidizes to form haematin, the staining agent. This oxidation process is referred to as ripening. A solution of haematoxylin in which oxidation has progressed to the stage where it produces only a weak stain is called unripe; one in which oxidation has progressed to the point where it produces a sharp, clearly defined stain, ripe, and an old solution that produces a diffused muddy stain because of over-oxidation, over-ripe. It will be seen that the oxidation process is progressive, resulting finally in a worthless solution. A well-ripened but not over-ripe solution is essential to the production of the well-defined differential stain necessary for critical microscopic study and photography.

If the solution is kept in a loosely covered container natural ripening takes place in from three to six weeks, depending upon the temperature. Oxidation is slow and the solution remains in good condition for from six weeks to three months if it contains only haematoxylin, or for many months if it is made up with alum according to the formula given here.

Haematoxylin may be ripened artificially by adding an oxidizing agent such as hydrogen peroxide. Several intermediate compounds seem to result from this addition, however, rendering the solution unstable so that it becomes over-ripe in a short time. Generally, natural ripening is to be preferred.

The next group of stains contains carmine. This is a deep red dye made from cochineal insects. It, like haematoxylin, forms colored lakes when combined with suitable mordants. These stain the nuclei a brilliant red or purple-red. Structures thus stained are brilliantly differentiated, yet quite transparent. For this reason car-

mine is the favorite stain for objects to be stained and mounted whole, such as aquatic forms, small insects and their larvae, flukes, tapeworms, etc.

The third group of stains consists of the aniline dyes in a large number of brilliant colors, of which comparatively few are of any use in microscopy. Safranin, for example, is a brilliant and powerful stain for nuclei, while eosin is the most precise and delicate stain yet discovered for cytoplasm, connective tissue and cell membrane.

Staining is a large field embracing many combinations of methods which it is impossible to cover completely in a work of this size. For this reason only general staining methods will be considered, with outlines complete and fully applicable to a large part of the material encountered by the student. For more detailed and amplified instructions one of the treatises mentioned in the bibliography should be consulted.

When working with histological material a nuclear stain is nearly always used first. Alum-haematoxylin is usually used, although some of the anilines give excellent stains for some material. Iron-haematoxylin is the most valuable stain for cytological preparations. Objects to be mounted entire are usually stained with carmine, with indulin as a counter-stain.

Since certain stains exhibit a selective affinity for some classes of tissue, it is possible, by a proper selection of stains, to color each class of tissue differently. Thus a chromatin stain may be selected for the nucleus, a plasma stain for the cytoplasm. Thus treated, the material will reveal the other tissues of which it is composed and appropriate stains may then be selected to differentiate the particular tissues to be studied.

The material to be studied, either sections or whole

objects, is gradually brought from the preserving fluid into the solvent used for the nuclear stain. If a water solution is used and the material has been preserved in 70% alcohol it is passed down through the descending series of alcohols until water is reached. If an alcoholic stain is used it is passed up or down the series as far as necessary, or until that concentration of alcohol is reached which equals the concentration of the stain.

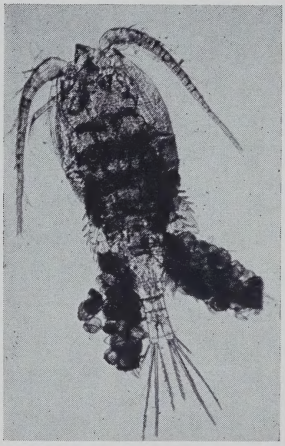


Fig. 35. Female Cyclops. An aquatic animal that can be reared in an aquarium. Fixed in Bouin's, stained in standard alum-haematoxylin, with indulin as a counter stain. X50

The material is now placed in the nuclear stain, filtered before use, where it remains until examination shows the nuclei to be deeply colored. Two methods are in use, one known as progressive staining, in which the material is stained in a dilute solution of the dye, the other called regressive staining, in which the material is first stained deeply in a strong solution and then destained in another reagent which extracts the excess stain. A more precise color differentiation usually results from the regressive method.

When staining has progressed far enough to insure complete impregnation, the object is transferred to a washing medium to remove adhering excess stain. This will be water or alcohol, depending upon the stain used; water for aqueous stains, alcohol for alcoholic stains.

The objects are now counter-stained by bringing them

up or down the alcohol series to match the concentration of the solvent of the counter-stain. They remain in this for a few minutes and are then rinsed a moment in water or alcohol as required by the counter-stain.

Dehydration of the material is accomplished by bringing it into absolute alcohol by graduated stages. If the counter-stain is one which washes out easily, dehydration must be effected as quickly as possible to retain the stain.

The dehydrated material is now ready for clearing and mounting. For details, see Chapter VIII.

The following formula for alum-haematoxylin is taken from Galigher. It is called by him "Standard Alum-Haematoxylin" and is a modification of Harris's formula. Galigher says of it, "it is a thoroughly satisfactory nuclear stain for general histological purposes."

STANDARD ALUM-HAEMATOXYLIN

Haematoxylin, white crystals	0.5 g
Aluminum ammonium sulphate	0.3 g
Alcohol (50%)	100 ccm
Mercuric oxide (red)	0.6 g

Dissolve the haematoxylin and alum with the aid of heat. When solution begins to boil add mercuric oxide and boil for twenty minutes. Then add enough 50% alcohol to make up the original volume. Allow to cool and stand over night. Then filter through two thicknesses of filter paper and stopper tightly.

This stain is ripened by the addition of mercuric oxide and is ready for use at once. Its staining power will increase slowly for a month or more. If properly stored, it will keep six months to a year.

When using this stain for sections the progressive process is preferable. The time and effort involved are re-

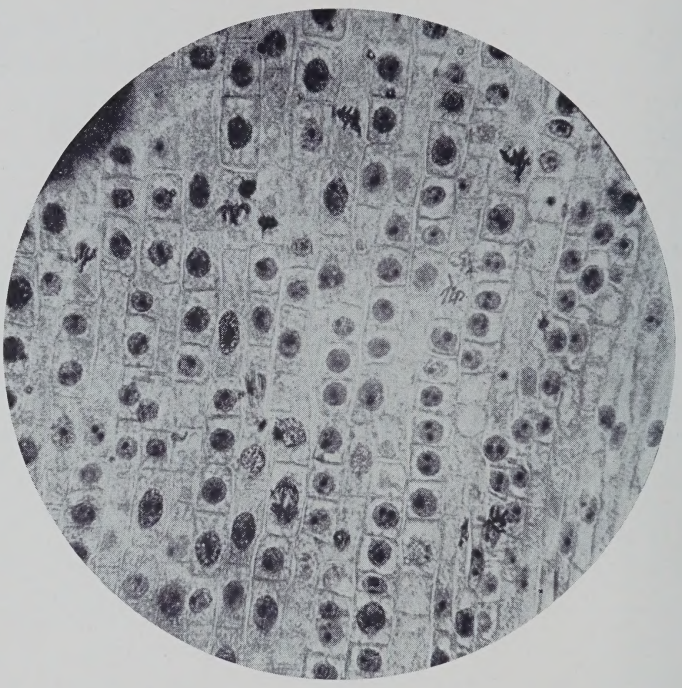


Fig. 36. Mitosis in onion root tip. Alum-haematoxylin stain to show chromatic figures and nucleus. No counter stain. X900

duced to a minimum, the depth of this stain is easy to control and the results usually equal those of regressive staining. Small and easily permeable whole organisms may be treated in the same way. The following directions apply to either class of material.

The solution used in progressive staining is made by diluting 12 ccm of standard alum-haematoxylin with 88 ccm of saturated solution of ammonia alum in distilled water. Allow the mixture to stand three or four weeks before using it. After this period of oxidation the staining power remains nearly constant for six to eight months.

1. Pass the material down the descending series of al-

cohols to water. Wash in water two or three minutes. If the material has been fixed to slides, such as paraffin sections, aquatic organisms or minute insects, set the slides in a pan through which water is running. Loose sections or free organisms may be handled by simply changing the water several times.

2. Transfer to staining solution and allow to remain about twenty minutes. Remove a piece of the material, rinse in water and examine under the microscope. If the nuclei are strongly stained, with the plasm colored slightly or not at all, remove the remainder of the material and wash for fifteen minutes in running water as in step 1. If the stain is weak, replace the test piece in the stain and at the expiration of ten to fifteen minutes examine again.

If a satisfactory stain is not achieved in forty-five minutes the solution is too weak or the material too impermeable. The stain may be tested for strength by trying another piece of material. If this test proves positive, stain the material by the regressive method; if negative add a few drops of standard alum-haematoxylin and immerse the material again.

The final washing in tap water serves a double purpose. First, it removes the last traces of alum which if left in the material would soon cause the stain to fade; secondly, the slight alkalinity of the water turns the stain blue.

3. Dehydrate as described earlier up to 80% alcohol. To the 95% alcohol and the first wash of absolute alcohol add enough eosin to color the liquid a deep pink. Leave until thoroughly counter-stained, wash quickly in absolute alcohol and place in the clearer.

To prepare standard alum-haematoxylin for regressive staining add one drop of concentrated hydrochloric acid to each 100 ccm of stain. Filter the solution before use.

1. Pass down through alcohols to water as in step 1. above.

2. Place in concentrated stain for twenty minutes.

3. Rinse material in distilled water and place in 0.5%

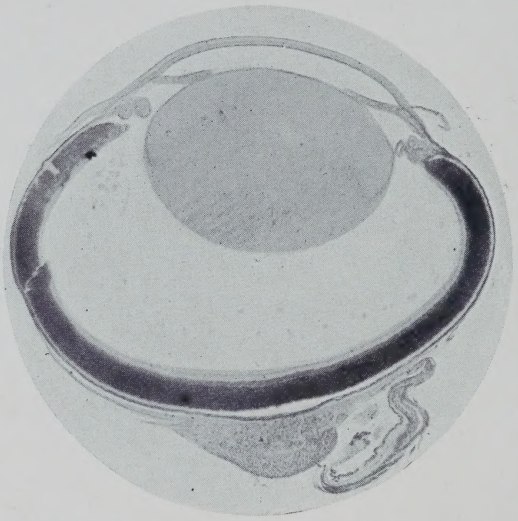


Fig. 37. Cross section through eye of rat. Stained with borax-carmine, whole. Photographed for contrast with the background. X50

solution of hydrochloric acid to remove excess stain. Watch material carefully and examine under the microscope frequently to ascertain the exact amount of stain remaining. Loose objects or sections may be placed in a watch glass on the microscope stage and observed throughout the process. When sufficiently destained transfer the material to tap water and wash thoroughly.

4. Dehydrate, clear and mount in balsam.

The carmine stains may be used after a large variety of fixing agents. Material fixed in Bouin's fluid or any of

the picric acid fixing agents stains beautifully with carmine, as does material fixed in alcohol and formalin.

Grenacher's alcoholic borax-carmine yields beautiful stains when used according to Lynch's method. For whole objects such as small Crustacea, Daphnia, some Polyzoa, Hydra, tunicate larvae, small Insecta, small flukes and the like it gives excellent results.

GRENACHER'S ALCOHOLIC BORAX-CARMINE

Carmine	3 g
Borax	4 g
Distilled water	100 ccm

Boil until carmine is dissolved, or better still, allow to stand until this takes place. Then add:

Alcohol (70%)	100 ccm

Allow mixture to stand several days and filter.

The procedure is:

1. Transfer material from 50% alcohol to stain and allow it to stand over night.

2. Cautiously add concentrated hydrochloric acid drop by drop, gently agitating the container, until all carmine is precipitated as a heavy, brick-red substance. After standing a short time the supernatant liquid must be a transparent red containing no trace of the deep translucent solution originally used. Allow to stand over night.

3. Add an equal volume of 3% hydrochloric acid in 70% alcohol to the solution in the container and agitate to mix the contents thoroughly. Allow material to settle and then decant or pipette off the carmine. Most of the precipitated carmine will remain in suspension and may be easily withdrawn. Examine the withdrawn liquid carefully for organisms that might have been drawn out

with it. If any amount of desired material is present place the liquid in a shallow dish, from which the wanted material may later be reclaimed. If no material is present discard the liquid. Refill the container with acid alcohol and allow it to settle, after which it must be drawn off again. Repeat the washing with acid alcohol until all precipitated carmine has been removed from the material.

4. Place the material in a watch glass with sufficient acid alcohol to cover it well, and observe under the microscope. The acid will slowly extract the stain and must be replaced with fresh solution as often as it becomes deeply colored. The destaining operation should extract most of the stain from cytoplasm and leave the eggs, gland cells, digestive tract, trachea, etc. deeply colored. Do not overdo the destaining or the residual tint will be too weak.

When destaining seems to have progressed far enough, pick out several organisms, place them in absolute alcohol for a few minutes, clear in creosote and examine carefully under a cover glass. In this way the staining of the bulk of material may be carefully controlled to give exactly the stain desired.

5. When properly differentiated, draw off the acid alcohol and replace with 80% neutral alcohol. Change this several times, depending upon the size and quantity of the specimens, in order to remove all traces of acid. Allow material to remain in last neutral 80% alcohol at least one hour.

6. Dehydrate, clear, infiltrate with balsam and mount.

When a solution composed of carmine, picric acid and ammonia is evaporated to dryness there results a reddish brown substance called picro-carmine, which is somewhat soluble in water. This solution gives a beautiful transparent stain to chromatin and is excellent for large flukes,

proglottids of tapeworms, and other forms in which the spaces between the organs consist of a dense parenchyma. It is equally well adapted to staining many aquatic forms, such as Paramecia, Volvox, Rotifera, Daphnia, Cyclops, etc., differentiating the organs with great clearness and precision.

> RANVIER'S PICRO-CARMINE — To a saturated solution of picric acid in water add a strong solution of carmine in ammonia until a precipitate begins to form. Add a small crystal of phenol to prevent development of fungi and let the solution stand in an open jar until evaporated to one fourth its original volume. Filter to remove precipitate and allow filtrate to evaporate to dryness. To use for staining make a saturated solution of the residue in distilled water and add a trace of phenol or salicylic acid.

To stain with picro-carmine pass the material down the alcohols to water, then transfer to the stain, which should be several times the volume of the material. Small aquatic forms should remain several days, larger forms a correspondingly longer period. When examination shows a satisfactory stain, wash the material in several changes of water, then up-grade to 70% alcohol. Change this several times at one-day intervals to remove the picric acid. Destain in 70% acid alcohol (0.5% to 1.0%) and examine frequently as directed under carmine staining. Destaining should be carried on until most of the color has been extracted from all structures except nuclei and the denser varieties of cytoplasm.

Dehydrate, counter-stain, clear and mount in balsam.

As has been mentioned, many of the aniline dyes are used both as nuclear and general stains, but their use in

the hands of the student is not likely to produce as satisfactory results as haematoxylin or carmine. Many of these dyes depend upon a delicate hydrogen ion concentration which is difficult or impossible to secure without a well-equipped laboratory. Hence only a few of the anilines will be considered. All of those mentioned have been used by the writer and worked out satisfactorily.

Eosin — This is one of a class of acid aniline dyes that is used quite generally as a counter-stain with haema-

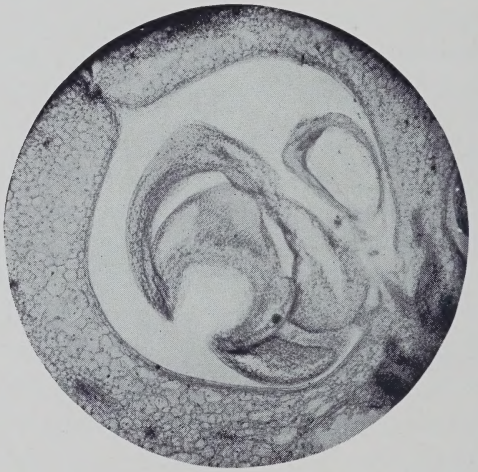


Fig. 38. Embryonic seeds in ovary of hyacinth. Alum-haematoxylin for nucleae, with eosin for plasm. X25

toxylin. It imparts a strong transparent color to cytoplasm, muscle fibers and intercellular structures, giving a valuable contrast with blue nuclear stain. When mounted in neutral balsam eosin is quite permanent.

The variety of chemical known as Eosin Y is a very good stain, easily soluble in water or alcohol. It is advisable to use an alcoholic solution because the aqueous

solution stains slowly, gives a rather diffuse stain and washes out easily. It is somewhat dependent upon the pH concentration for best results. A 0.5% solution in 90% alcohol is adjusted to a hydrogen ion concentration of 5.4–5.6 by adding 3.2 ccm of .1 N hydrochloric acid per hundred ccm of dye solution. This should result in the solution which will give the best results.

Eosin may be used following formalin or Bouin's fluid for fixing, the latter giving better preservation and excellent staining.

INDULIN — A blue dye which provides a beautiful counter-stain for whole objects stained with carmine.

Fig. 39. Spiral vessels from stem of rhubarb. Malachite green stain. Mounted in glycerine jelly. X100

The product marketed by Grübler under the name "Indulin Grünlich, nach Rawitz" is the only really dependable one. American-made indulins have proved untrustworthy. It is a valuable stain for bringing out clearly such appendages as cilia, bristles, spines, flagella, etc.

Once in the tissues, this dye is difficult to remove, hence

it is used as a progressive stain in very dilute solution. Prepare for stock a 0.16% solution of the dye in 90% alcohol. When ready to use it, add enough stock solution to the 90% dehydrating alcohol to color it lightly. Immerse the material and examine it frequently, as the stain acts quickly. When the desired depth of color is attained, transfer the material immediately to clear 90% alcohol. Be sure the alcohol used is neutral or slightly acid, never alkaline, as the slightest trace of alkali will prevent staining or produce a degraded color.

Malachite Green — A deep green aniline dye, soluble in water or alcohol. It is useful for staining vegetable sections or teased material, aquatic forms, insects and larvae.

To prepare the stock solution take:

Malachite green	2 g
Distilled water	90 ccm
Glycerin	30 ccm

Mix water and glycerin and dissolve the dye.

To use add enough stock solution to distilled water to color it strongly, and filter. Immerse the material until stained. Destain in 50% alcohol acidulated with 0.5% acetic acid.

Soluble aniline blue in a 2% aqueous solution, filtered, may be used for leaf hairs, epidermis, vegetable sections, etc., in the same way as malachite green.

Chapter VII

Preparing and Mounting Hard Objects

When hard animal structures such as bone, teeth, horn, nails, etc. are to be studied, methods different from any thus far discussed must be used to secure thin sections. Minerals, too, require a different technique, as will be described later.

Since bone is easily procurable and a very interesting object for the microscope, especially the polarizer, let us first examine the methods of sectioning it for study.

Two methods of preparation are in use, one a grinding process in which the natural bone is reduced to a thin section in the dry state, the other a decalcifying process in which a reagent is used to soften the bone to the point where sections may be cut with a knife. Both methods have their adherents, so it will be well to describe both.

Grinding Sections of Bone — Use only fresh bone that has been placed in water as soon as the surrounding soft parts have been removed. Bone that has been permitted to dry naturally does not give a true view of its character because of infiltration of fat from the medullary canal which takes place as fast as the water evaporates. The same is true of bones that have been boiled. Therefore, procure perfectly fresh material and immerse it in water as soon as possible. Cut the bone while wet into pieces as thin as possible, using a hack saw and the finest toothed blade procurable. Cut both transverse and longitudinal sections and immerse them in water, where they remain until all soft tissue has unquestionably dissolved out or corroded away. No other reagent need be used,

but plenty of time, several months, is required to secure proper cleaning. Change the water from time to time, shake the contents occasionally and, when all soft parts have been destroyed, wash the bone thoroughly with clear water and allow it to dry.

Take the section of dried bone, place it on a fine-cut file and rub it back and forth, first on one side, then on the other, until it is quite thin. Change to very fine emery or garnet paper and continue grinding until the section seems to be so thin that it will transmit light. Examine it under the microscope and you will be surprised to find that it does not transmit sufficient light to reveal details, but is merely translucent. Now transfer the section to a stone hone and continue grinding until the section is extremely thin and transparent when examined with the microscope. Do not press hard while grinding for the pressure cannot be distributed equally and some spots will be ground thinner than others. Furthermore, it is very easy to break the thin sections. Try to keep the surface level by uniform grinding. Bone grinding is a slow, tedious process, but careful work is certainly justified by the beauty of the specimen when well prepared.

When the section is thin enough, put it into a test tube or shallow vessel and wash with a gentle stream of water from a pipette to remove bits of ground bone. When clean, place it in absolute alcohol for a few minutes, then lay it between two slides and let it dry thoroughly.

Take a small quantity of regular balsam and drive off a good part of the solvent with gentle heat, until a drop of it solidifies as soon as it cools.

Put a small piece of this hard balsam on a slide and another piece on a cover glass. Warm both of these until

the balsam melts and spreads slightly, then allow to cool until solidification takes place. Place the ground bone section on the balsam on the slide, lay the cover glass on the section, balsam side down, and warm the slide very carefully until the balsam begins to melt. Quickly press the cover down on the section with some soft, blunt instrument, to spread the balsam to the edges of the cover. If properly done the balsam will infiltrate the bone matrix and render it transparent, while the Haversian canals, canaliculi, and lacunae will be left filled with air. Because of the difference in refractive index these will seem black against the white background of the bone, thus making greater contrast. If the balsam is too warm it will enter the cavities and make them transparent, thereby destroying the structure of the bone.

Decalcified osseous tissue may be cut in the microtome like paraffin sections, and if properly done serves the same purpose as ground sections without entailing the tedious grinding of the dry process.

Hydrochloric acid is the most widely used decalcifying agent. It is not the best to use because it makes tissues swell badly. Nitric acid does not do this. However, nitric acid in strong solutions exerts a powerful gelatinizing action on bones, which may fortunately be controlled by the addition of alcohol. Busch (Lee's Vade-Mecum) recommends the use of one part of concentrated nitric acid in ten parts of water. Fresh bones are placed in 95% alcohol for several days and then transferred to the nitric acid, which is changed every day for eight or ten days. The specimens must be removed as soon as they are decalcified or they will turn yellow.

Another very excellent decalcifying reagent is 3% nitric acid in 70% alcohol. Bones should be softened in

Fig. 40. Forming bone in the foot of a rat. Alum-haematoxylin and eosin. Decalcified for cutting sections. Approximately X100

this mixture for from several days to a week or more, depending upon their size. The liquid must be changed every other day. As soon as a needle can be thrust into the bone it is soft enough to cut. Remove from the acid alcohol, wash in 95% alcohol and place in 95% alcohol containing an excess of precipitated chalk to free the material from all traces of acid. Wash until the alcohol fails to give an acid reaction to litmus paper. Place in absolute alcohol to dehydrate, clear in toluol and infiltrate with hard paraffin. Sections may be cut in the same manner as paraffin sections, and may be stained in the same way.

Transverse and longitudinal sections of teeth make very interesting slides, but the difficulties in preparing

them are great. The student should know how it is done even though he does not make actual sections. Thin slices are cut from the whole tooth with an emery saw. This consists of a thin piece of flat sheet metal, such as a strip of clock spring, or even a piece of tin. The cutting is done by flooding the work with water in which is suspended powdered emery. First make a scratch on the tooth's surface with a metal-cutting saw to afford a starting place for the cut, then start the emery saw, using it exactly as you would any saw. Using plenty of water and emery, work the metal strip back and forth and in a short time the cut will be made. The particles of emery take the place of saw teeth and cut rapidly. New emery must be added as rapidly as it is used up. If only one section is to be made from a tooth, considerable time may be saved by grinding the tooth down on an emery wheel until the place is reached where the section is to be made. Then only one cut with the emery saw is required.

The section is now reduced to the requisite thinness by rubbing between two pieces of plate glass. If the sawed section is thick and requires considerable grinding to reduce it, emery powder and water are used between the plates. If the sawed section is thin, use finer powder. When reduced to comparative thinness, wash the plates and the section with water, substitute pumice powder for the emery and continue grinding. When reduced to almost the proper thinness, again wash plates and section in water, then continue the grinding with rotten stone. Examine the section from time to time and when the numerous minute scratches have disappeared, wash again and replace the rotten stone with precipitated chalk and water. When examination shows that the section is thin enough to reveal all details and all scratches have been

polished out, remove to 95% alcohol, then to absolute alcohol to dehydrate, when it is ready for mounting.

Sections of teeth seem to suffer some loss of fine detail when mounted in balsam, so they are usually mounted dry, with gum acacia. Make a solution of the gum in water by dissolving 5 g of the dry powder in 100 ccm of distilled water. To this add 6 drops of glycerin and 25 ccm. of 95% alcohol.

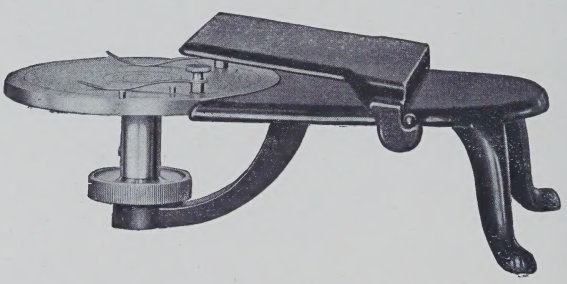


Fig. 41. Turntable for ringing slides and turning cells.

Spin a ring of gold size on the slide in the turntable. When this is dry, paint a thin film of gum water on the slide inside the ring and allow this to dry thoroughly in a place free of dust. A desiccator is a very good place to dry slides. When perfectly dry, take the section of tooth out of the 95% alcohol, place in absolute alcohol for several minutes, and permit the alcohol to evaporate. Breathe lightly on the gum film on the slide, quickly set the section in place in the center of the ring, and press into contact. The condensed moisture from the breath will soften the gum enough to make it adhesive and the section of tooth will adhere to the slide. Set in the desiccator until dry, spin another ring of gold size over the first one and quickly set the dry cover glass.

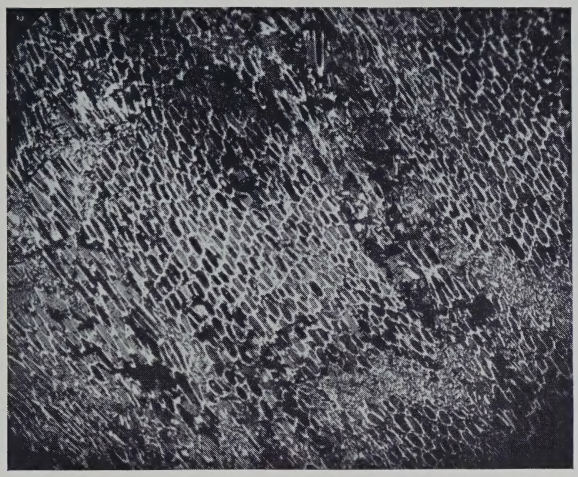


Fig. 42. Section through coal. Ground section, showing original cell laminae. X50

Coal, being of vegetable origin, affords opportunity for the preparation of very instructive slides. Not all samples of coal will reveal the woody character, but some pieces may be found to display a most interesting structure. Generally the softer grades of anthracite or the harder bituminous coals afford the best material. Sections may be procured either by grinding or by cutting.

Grinding Sections of Coal — To grind sections of coal first cut thin slices with a metal-cutting saw or the emery saw. Then make one side of the section smooth by rubbing first on a file, then emery paper, then crocus paper laid on a piece of plate glass. Use the finest grade of paper available in the last stage of paper grinding. Now transfer the section to a hone, using plenty of water, and grind down on this until perfectly smooth and free from scratches when examined with the microscope. Scratches that are invisible to the unaided eye will show

up like deep valleys under the microscope. When the section is perfectly smooth, it must be polished by rubbing on a dry piece of fine linen stretched taut on a piece of plate glass, using jeweler's polishing rouge as the agent.

The polished section is placed on a slide, polished side down, by putting a drop of hard balsam on the slide, warming until the balsam melts and spreads, and pressing the section into contact. When cold, the section must be firmly attached to the slide at every point or it will break out at the free places when it is reduced to the required thinness.

Because of its color, coal must be ground thinner than light-colored objects, to reveal its structure. If the section attached to the slide is not very thin, it may be reduced quickly by filing. If already thin, proceed at once to the fine emery paper and crocus paper, grinding the free side of the coal. As the section becomes thinner examine it frequently with the microscope until the details begin to appear. Then transfer the slide to the hone and work it smooth, using plenty of water. Keep honing until the section is reduced to such a thickness that the details are clearly discerned. Now transfer to the polishing cloth and polish the specimen. Work slowly to avoid heating the slide enough to soften the balsam and dislodge the section. When polished, transfer to clear water and wash thoroughly to remove all traces of abrasive. Dry the slide and section thoroughly in the desiccator or oven. When perfectly dry, add a drop of xylol-balsam and a cover glass to protect the specimen.

The foregoing method of grinding hard specimens applies to all minerals and such animal subjects as Echinodermata, shells of Mollusca, corals, fossil wood, etc.

Cutting Sections of Coal — "The Micrographic

Dictionary " recommends a process by which coal is rendered sufficiently soft to permit section cutting with a razor. The author has had no experience with this method, which is somewhat vague in that the strength of the macerating fluid is not given. If the student wishes, however, he may start experimenting with a 25% solution. If this is too energetic, or too weak, alterations in strength will be indicated and the solution may then be adjusted either way to give the desired results.

The method given advises macerating the coal for a week to ten days in a solution of potassium carbonate. At the end of that time it should be possible to cut moderately thin sections with a razor. These are then placed in strong nitric acid, covered and heated gently. They soon turn brownish, then yellow, when the reaction must be stopped immediately by throwing the whole into a large volume of clear water, or the coal will be dissolved. The sections should be dark amber and very transparent, and should exhibit the structure clearly. Sections thus prepared are best mounted in glycerin jelly, as balsam seems to make them more or less opaque.

This opacity resulting from balsam mounting is probably due to water of inclusion. The remedy may be found in complete dehydration in absolute alcohol, followed by clearing in toluol and mounting in balsam. As previously noted, balsam is the best mounting medium for most objects and every effort should be made to bring them into condition where it can be used. Absolute dryness is essential, for the least trace of water will cause the balsam to become cloudy and the specimen more or less opaque.

Friable objects such as calcareous shells should be infiltrated with balsam before grinding. Saw thin sections

of the material and immerse them in xylol for a week to insure complete penetration of the solvent. Then place in thin balsam and allow this to evaporate until it is quite thick. Place on a slide and warm gently to drive off the remaining xylol and harden the balsam. When hard, grind and polish the exposed face of the section. Wash thoroughly, dry and warm the slide just enough to allow removal of the section. Place the polished face in contact with the slide and fasten with balsam. Now grind to the required thinness, wash, dry and mount in balsam.

Chapter VIII

Preparation of Animal Material

We have already examined some of the microscopic members of the animal kingdom in our study of aquatic organisms. Now let us turn our attention to another prolific source of material for the microscope, as represented by the larger forms of life with which we are more familiar. This class includes all animal tissues, such as nerves, muscles, teeth, horn, bones, feathers, hair, skin, etc., and that group which provides such a wealth of microscopic material, the insects.

The group of animals scientifically called Arthropoda is the largest biological group in existence. It contains more families, genera and species than any other group. It is the most widely distributed geographically, and exhibits more diversity of form, habitat and life history than any other group. Since this group will provide the student with the greater part of his microscopic material, let us digress for a moment and examine these creatures more carefully.

The Arthropoda are distinguished from all other animals by the fact that their bodies seem to consist of a series of rings or segments, apparently joined together, and bearing certain hard jointed appendages. Examine a caterpillar under a hand lens. You will see that it consists of a long cylindrical sack which is folded in upon itself at regular intervals, giving it an articulated appearance. Some of the arthropods, such as the true insects and the Crustacea (crabs, lobsters, crawfish, etc.) have what we call an exoskeleton, that is, the skeleton is on the outside

of the body instead of on the inside as it is in humans, cattle, dogs, etc. This exoskeleton is composed of a hard, horny material called chitin, which, when the animal dies and dries, breaks readily into perfect rings that clearly reveal the articulated structure. It is by the grouping and arrangement of these rings, as well as by the appendages (legs, antennae and wings), that the various groups of

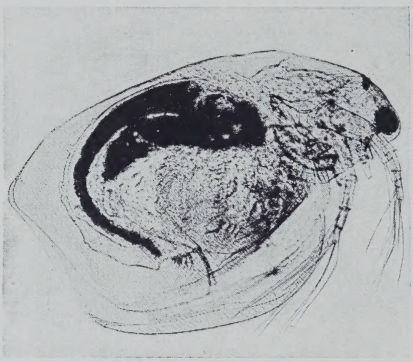


Fig. 43. Fresh water crustacean. X70. Slide and photograph by Irving L. Shaw.

Arthropoda are classified. The true insects, for example, are called Hexapoda because they are provided with six feet, terminating as many legs, all of which issue from one part of the body called the thorax. An animal belonging to the group loosely referred to as bugs is not a hexapod if it has more or less than six legs. Nor is the specimen necessarily a bug, for the true bugs are a separate group of insects, known correctly as Hemiptera. Spiders are considered insects and are included in the Arthropoda, their specific classification being Arachnida.

This division of body segments for the performance of specific duties may be observed in the larval stage of all true insects. For example, any small, elongated, tubular-

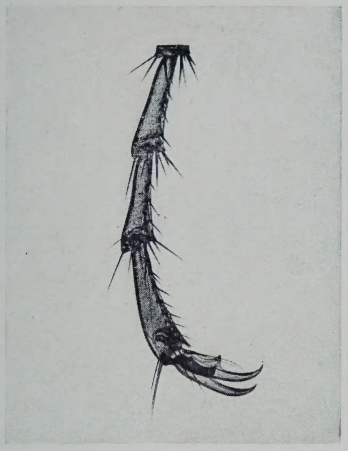


Fig. 44. Foot of beetle. X18

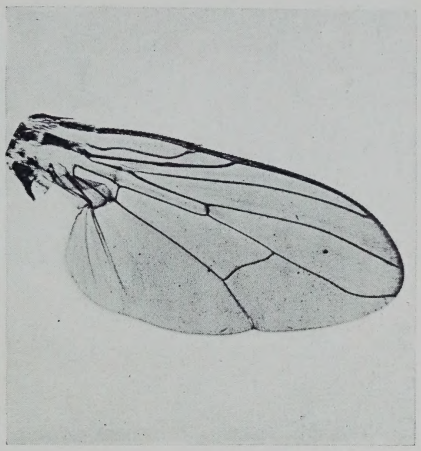


Fig. 45. Wing of house fly. X13

Photographs by Irving L. Shaw

bodied crawling animal is to many people a worm. Scientific classification is not so loose. The true worms have been classified into one order, the Vermes, distinguished by specific relationships. While the second or larval stage of insects may resemble worms in general, there are very pronounced differences apparent to even the casual observer. While the bodies of both are articulated, in the insect larva certain segments have certain duties to perform. Let us examine the caterpillar (larva) of any moth or butterfly, preferably one without hairs and a large one in which the segments may be easily seen. Such specimens may be found feeding on cabbages, potato vines, sassafras leaves, tomato stalks and any number of similar places. Almost every plant has an insect larva that feeds upon it in preference to any other, so it is not difficult to find suitable specimens.

The first segment of the body is always the head, dis-

tinguishable by the presence of large eyes and a horny texture different from that found anywhere else on the body. The second, third and fourth segments will each bear a pair of legs bilaterally placed, the true walking legs of the creature. These are hard and terminate in hooked feet visible under low magnifications. Further along toward the posterior end of the body of some larvae are found the pseudopoda (false feet) or claspers. There are ten of these, two each on the seventh, eighth, ninth and tenth segments, with the fifth pair on the last or thirteenth segment. This pair of claspers may be modified to bear but slight resemblance to the other eight, yet its purpose is exactly the same. The pseudopoda are distinguishable from the true feet in that they are a great deal larger in diameter, soft and pulpy in texture and flat on the ends. They are used not as feet, to propel the animal, but as a means of taking hold of a twig or branch while the head end of the body swings about in the air in search of a new feeding place. This phenomenon may frequently be seen among larvae such as the so-called measuring worm, a green caterpillar that humps itself up in the middle as it travels along, then stops and rears itself on its pseudopodae, holding the forward part of the body nearly erect while the head waves about hunting a feeding place.

When the insect undergoes the third stage of its life cycle, called the pupal stage, in which it remains dormant for a period, the three anterior leg-bearing segments are, in a manner of speaking, fused into one. This, the middle portion of the body of an adult insect, is the thorax, from which issue the six legs that distinguish the order. This distinguishing characteristic may be found in every true insect, including the Diptera (flies), the Lepidoptera (butterflies and moths), Hymenoptera (bees, wasps, hor-

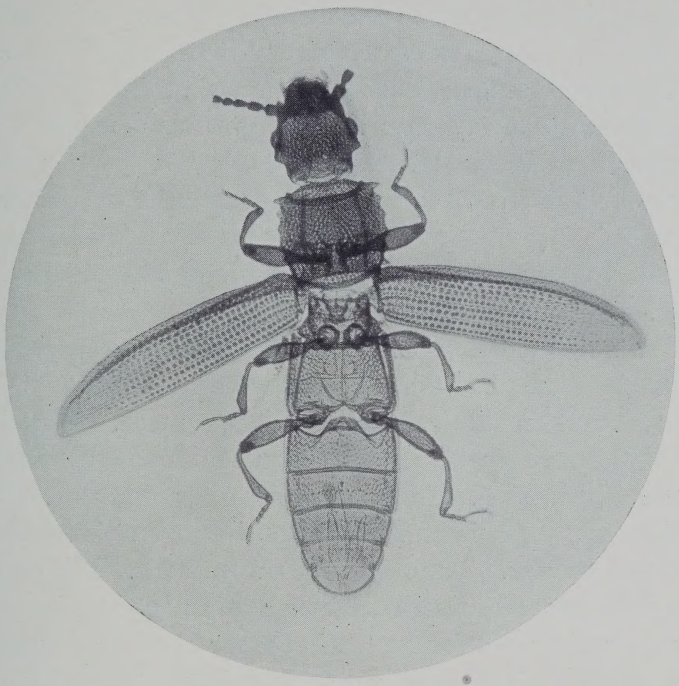


Fig. 46. *Sylvanus surinamensis.* Balsam mount. Photographed to show detail. Note female reproductive organs at anal end. X35

nets, etc.), Orthoptera (grasshoppers) and Neuroptera (dragon flies). In the order Coleoptera, which includes the beetles, the thorax is extended to form the prothorax, which carries two pairs of legs, while the thorax carries the anterior pair. This arrangement of legs is well illustrated in the photomicrograph of the beetle *Sylvanus surinamensis,* Fig. 46.

Any one of the insect orders will provide the student with abundant material for study. The order Lepidoptera, for example, is rich in a diversity of material. All moths and butterflies lay eggs which may be found by dili-

gent search among the leaves of the specific food plant of the species. Each insect prefers one particular plant on which to feed, and since it is the larva that does the feeding, the adult always lays its eggs on that plant so that suitable food is immediately available when the young larvae emerge from the eggs. Of course the beetles and bugs, as well as the butterflies, lay eggs which may be found attached to the surface of food-plant leaves or stems.

These eggs exhibit a variety of forms, one of which is shown in Fig. 76. These are the eggs of *Podisus spinosus,* found on the leaf of a wild cherry tree. Most insect eggs have some form of ornament such as the spines in the illustration. In one species they resemble tiny mushrooms, while in another the entire surface is studded with tiny bumps. Some eggs are transparent. If the student is fortunate enough to secure some of these he can place them in a cell for protection and watch the development of the embryo within the shell from day to day.

The next stage of insect development is the larva. This is the feeding stage, in which most insects do their destructive work. Many adults feed voraciously, especially some of the beetles, such as the Japanese beetle (*Popillio Japonica*) , and the rose chafer (*Macrodactylus subspinosus*) and the potato beetle (*Doryphora decemlineata*) , to mention only a few commonly-known species. Others, particularly the moths and butterflies, feed little or not at all during the adult stage, and then only on nectar which they obtain from flowers, for their mouth parts consist only of sucking tubes. These are curled up into a close spiral resting between two extensions on the head called the maxillary palpi. The adult exists only to propagate the species. This the male does by fertilizing the

eggs which the female then deposits on the appropriate food plant.

The larval stage of insect development is of interest to the microscopist because of the opportunity it affords to study the anatomy of the caterpillar. Many of the larvae are transparent, or at least translucent. If one is placed on the microscope stage many of the body functions may be observed in life. Others are colored green by the chlorophyl of the leaves they eat. Usually this green color can be extracted with alcohol, when the alimentary canal will become visible through the skin of the larva.

An interesting experiment may be performed on some caterpillars to show the course of the alimentary canal, the circulatory system and the respiratory system. It is delicate and will require considerable care, but successfully accomplished is well worth the effort. It consists of injecting into the body solutions of stains to color each system differently so that they may be easily discerned.

The best subject is a large caterpillar with a smooth skin, such as the larva of the tomato hawk moth (*Macrosila quinque-maculata*). This may be found feeding on tomato and potato vines. Examine it carefully with a hand lens to find the mouth opening, then examine the sides of the larva to locate the spiracles or openings to the breathing tubes.

Three stains will be needed, as well as injection apparatus of some sort. The subject is small so will not retain much fluid, ten ccm of each being quite sufficient for several specimens.

For the alimentary canal use:

Acid aniline green	0.5	g
Distilled water	100	ccm
Glycerin	35	ccm

Add glycerin to water and dissolve acid aniline green in this to make a stock solution. For use take 5 ccm of stock solution to 25 ccm of distilled water.

For the circulatory system use:

Carmine	3	g
Borax	4	g
Distilled water	100	ccm

Dissolve borax in water and add carmine. Heat gently to dissolve, then add 100 ccm 70% alcohol, filter and keep well stoppered.

For the respiratory system use:

Methyl violet	0.5	g
Distilled water	100	ccm

The best form of injection apparatus is a hypodermic needle. Lacking this a satisfactory substitute may be made by drawing glass tubing out to a very fine point in the flame and equipping the pipette thus made with a small rubber bulb such as is used on medicine droppers. In fact, medicine droppers may be used for injections if the points are drawn out to a fine needle shape. A separate pipette should be provided for each stain so that one color is not contaminated by another. Fill each pipette and lay them ready to hand so that they may be used in quick succession.

Have everything in readiness. Grasp the caterpillar firmly in the left hand but do not crush it. Insert the tip of the injector containing green stain into the mouth of the larva and compress the bulb. Proceed very gently and continue the injection until the stain is forced out of the anal end of the specimen. Now that the alimentary canal is stained, proceed to inject the circulatory system with red stain by puncturing the back of the larva until

the tip of the injector just passes through the blood tube. This is the most delicate part of the operation and great care must be taken not to puncture both walls of the tube or the stain will enter the body cavity and fill it, thereby destroying the effect. The blood tube, which in insects takes the place of the heart in humans, lies directly under the skin on the dorsal side, running directly through the middle of the back from end to end. If the blood tract alone is stained it will appear as a thin red line running along the back. Sometimes, although by no means always, the alcohol in this second staining kills the caterpillar. In either case proceed at once to stain the respiratory system by injecting violet stain into the trachea or spiracles, as the breathing pores along the sides are called. Inject one spiracle and observe the result. Sometimes it is necessary to make a separate injection into each opening because of blocking of the tubes, and each side must be injected separately since there are no interconnecting tubes between the two sides. This injection will kill the specimen if not already dead because it shuts off its air supply.

When all three systems have been injected place the subject in 50% alcohol until the natural green color is gone, when the injected systems should be clearly discernable. If the subject is not entirely clear and transparent it must be dehydrated slowly by passing it up through a series of graded alcohols until absolute alcohol is reached. To do this remove it from the 50% alcohol and place it in 70% alcohol for about two hours, then in 80% alcohol for the same length of time, then in 95% and finally in absolute alcohol, where it must remain until it is completely free from water. Any trace of water will make it impossible to clear properly. Leave in absolute alcohol one hour if a medium specimen, two to three hours for large ones.

It might be well to explain the reason for the graded series of alcohols. Alcohol has the power to extract water from material placed in it. Since somewhere in the neighborhood of 98% of all animal matter is water this means that the supporting cells of animal matter retain their shape largely because of their water content. If this is removed too rapidly the material will shrink unequally, resulting in a wrinkled appearance which bears no resemblance to the subject in life. On the other hand, if the water is extracted slowly, the tissues shrink somewhat, but the progress is more uniform and the relative positions are retained in a more naturalistic arrangement, being fixed by the alcohol as it extracts the water.

When the specimen is completely dehydrated it must be cleared by replacing the alcohol with a chemical to render the tissues transparent. For this we use a mixture of equal parts of xylol and beechwood creosote. This has the property of penetrating the tissues and making them transparent, while at the same time it is soluble in Canada balsam so that the specimen may be infiltrated with balsam for preservation if desirable. Pour off the absolute alcohol and quickly replace it with creosote-xylol in which the specimen must remain until it is perfectly clear and transparent. If there are any whitish or opaque spots the subject was not completely dehydrated and must be returned to absolute alcohol for another period and again cleared. When it appears to be perfectly cleared pour away the used creosote-xylol and replace it with fresh solution, in which the specimen may remain for several hours, when it will be ready for infiltration with Canada balsam or paraffin. For either of these steps consult the chapters in which they are described.

Should the specimen be entirely satisfactory after it has

been bleached in alcohol to remove the chlorophyl, immerse it in distilled water over night to remove the alcohol, when it may be preserved indefinitely by imbedding in glycerin jelly. To make this take:

Gelatin (U. S. P. grade)	8	g
Distilled water	52	ccm
Glycerin	50	ccm
White of egg	5	ccm
Carbolic acid crystals	0.1	g

Soak gelatin in water for one hour, then dissolve with gentle heat. Add glycerin and white of egg, stir until thoroughly mixed and heat to 75° C. for 30 minutes. Filter through flannel while hot and add carbolic acid to the filtrate. Store in well stoppered bottles. This must be warmed for use to make it fluid.

To imbed a specimen in glycerin jelly soak the subject in water until every trace of alcohol is removed. Warm the jelly just enough to make it fluid. Place the specimen in a shell vial and fill to the top with warmed jelly. Stopper well and dip the neck in melted paraffin to seal. Thus prepared the specimen will keep indefinitely.

Insect larvae may be prepared for sectioning by hardening the fresh subject in alcoholic Bouin's fluid, the formula for which may be found in Chapter III. This will harden in about three days and will render the material in good condition to cut paraffin sections. If more rapid hardening is necessary for quick sections on routine examination work, harden in formalin 100 ccm, water 600 ccm. This will harden in two days, but the cutting quality of the specimen is not as good as that of Bouin's fixed material. After fixing, imbed in paraffin and cut sections as explained in Chapter V.

Dissection is an important operation in the preparation of microscopic material and the study of insects gives the microscopist an excellent opportunity to become familiar with it. Insects are abundant, so no fear need be entertained of ruining a specimen through clumsiness in the first dissection. Some specimens will necessarily be sacrificed in the process of acquiring skill in the use of dissecting needles. Working with fine needles under the microscope is quite different from performing the same work using both eyes. First, the three dimensional effect is lacking because of the monocular vision. Then, only a very small part of the specimen is seen at one time and it is somewhat difficult to direct the movements of the needles in the small field covered by the lens. If a lens of the triple aplanat or simple magnifying type is used the image will be right side up, as seen by the unaided eye. This is also true of the binocular microscope of the Greenough type. But if the dissection is carried out under the low powers of the compound microscope the work is rendered more difficult because of the inverted image presented by this instrument. Right becomes left and up becomes down, movements that are difficult to coordinate at first.

A dissecting microscope of some sort will be necessary for dissection of small parts of insects, vegetable fibers, animal fibers, etc. Such an instrument need not be elaborate or expensive, as long as the optical system is good. The lens may be either simple or compound, depending upon the magnification desired. Powers of ten to sixteen are ample for general student work. The lens should have a flat field, the largest field that the desired magnifying power will permit, and the longest working distance commensurate with its power. All of these requirements

are fulfilled by the type of lens known as a triple aplanat. This is somewhat expensive compared with simple lenses, but its corrections are so good that it is the ideal monocular lens to use for dissecting work. The only improve-

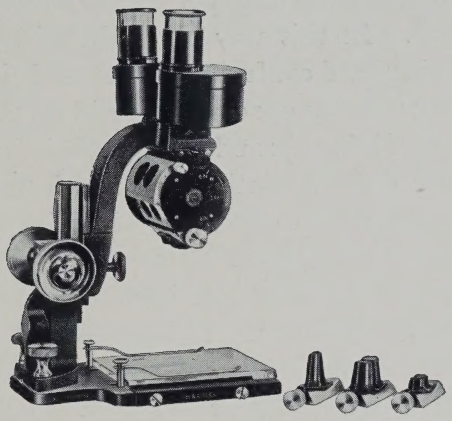


Fig. 47. Bausch & Lomb Greenough type Binocular Microscope.

ment over this type of lens is the Greenough type binocular microscope, an expensive instrument carrying two objectives and two eyepieces, which is the most comfortable microscope to use for dissecting and examination of whole objects in general. The image is presented to the eye erected and, because of the binocular arrangement in which the subject is viewed with both eyes, the effect of perspective is excellent.

A support of some sort must be provided for both the lens and the subject. In the absence of a proper dissecting microscope these may be improvised, the only requirement being that the lens be held in a fixed relation to the subject so that it will stay in focus. Some provision should be made for illumination of the subject from below, a valuable aid in many cases of dissection. Some material may

be satisfactorily manipulated in reflected light. In either case, there should be enough light to avoid working in semi-darkness, but not enough to be glaring. An excellent way to provide a pleasant working light is to set up a screen of tracing cloth or paper between the light source and the work. In this way the light will be diffused evenly, avoiding hard confusing shadows. It is also an excellent plan to wear an opaque eyeshield on the forehead, to intercept the light rays and prevent their falling on the upper surface of the dissecting lens and introducing disconcerting reflections. Occasions will arise when a concentrated light is necessary. This may be provided by introducing a condensing lens into the light beam and focusing it upon the work. This purpose is served very nicely by an ordinary Florence flask such as is used in chemical laboratories. The flask may be filled with water colored to give any type of light desired. Alternatively, a plano-convex lens may be used.

Many dissections may be successfully carried out under the lens of a tripod magnifier such as is sold for linen testing. This lens has a magnifying power of seven to seven and one half times. It is uncorrected for color and flatness of field, but it has a long focal length which permits free working with the needles under it. The self-contained legs permit its use in trays or directly on the body of large specimens. One serious disadvantage is the presence of the three legs, which sometimes interfere with free manipulation of the needles, but extreme portability and low cost help to compensate for this. In Chapter XI several improvised dissecting microscopes are described, any one of which may be procured at low cost.

The tools for dissection are simple to make and easy to procure. First a set of needles will be needed. In their

simplest form these consist of fine and coarse needles thrust into wooden handles, as described in Chapter XI. We will also require two pairs of forceps, one with fine curved points and one with strong blunt points. Fine-pointed scissors that cut at the very extreme tip of the blades are needed, also a scalpel or lancet. Numerous improvisations of the last item may be made from safety razor blades, but none is quite as satisfactory as a surgical instrument. Considering the low cost of a really good scalpel and the pleasant working it affords, it is false economy to attempt to work with substitutes. The Bard-Parker company make a line of low priced surgical knives which serve the purpose splendidly. The instruments are arranged so that the blades may be removed from the handles in a manner similar to safety razors. The handles are bought separately and the blades are available in several styles. The number 11 blade, made to fit the number 3 handle, is good for general use, while the collection may be completed by the addition of numbers 10 and 12, the former a curved blade, the latter scalpel shaped. The number 4 handle is much larger and takes larger blades, and will seldom be needed.

The last piece of equipment needed is a shallow tray or dish in which to perform the dissection. Most dissections should be carried out in a fluid of some sort, physiological salt solution being generally preferred. A good selection for this is the type of glassware known as a Petri dish, a shallow cylinder with a flat bottom. These are purchased in pairs, one of which acts as a cover. Very large specimens may be handled nicely in glass photographic trays. The requirements are few; the receptacle must be watertight, large enough to contain the specimen easily, and preferably transparent for substage illumination.

In some dissections portions of the specimen must be held rigidly while work is being done on another part. Since both hands are needed for manipulation of the dissecting needles, the subject is transfixed with needles thrust into the bottom of the dissecting tray. This means that the bottom of the tray must have a soft lining. Such a lining may be added to any glass tray by fastening strips of thin cork to the bottom with shellac. Cut strips of gasket cork (procurable from auto accessory stores) one sixteenth of an inch wide, using a sharp razor blade and a straight-edge. Paint one side with thick shellac and fasten to the bottom of the tray, allowing about one quarter of an inch between them. Next cut short strips of cork to lengths that will just fill the spaces between the long strips and shellac these in place, forming a lattice on the tray bottom, which will permit fastening the subject to the tray with needles. It may be illuminated by the substage mirror, and both hands are free for work. Large dissections that need not be performed under the lens may be carried out in a tray lined with cork shellacked to the entire bottom. In this case the light must be arranged to cast a minimum of shadows, and plenty of light must be provided.

The physiological salt solution referred to for use in dissections is made by dissolving table salt (not the iodized variety) in water. Six-tenths of a gram in one hundred ccm of water is the correct concentration. If a larger quantity of solution is required it may be made by dissolving six grams of salt in one liter of water.

Most insects, in fact all but butterflies and moths of which the wing scales are to be mounted, may be collected in alcohol when wanted for dissection. Use the ordinary 70% rubbing alcohol and drop the specimens into it as

they are collected. If they are being collected for sectioning they should be killed in cyanide gas. Potassium cyanide is a deadly poison and must be kept out of reach of children. Place a small quantity of the dry salt in the bottom of a wide-mouthed bottle or jar fitted with a tight cover. A layer one-eighth of an inch deep is ample. Over this place four layers of blotting paper cut into discs that fit closely into the jar. Force these into place with a stick and close the jar. It is ready for use at once. Drop the insects into the jar and close the cover. The gas arising from the cyanide (hydrocyanic acid gas) will kill insects in a few seconds, leaving them little time to beat about and destroy their wings. As soon as the specimens are dead they must be removed from the jar and spread if they are to be preserved as whole objects, or placed in Bouin's fixing solution if they are to be sectioned. Specimens intended for dissection may be preserved in alcohol, or they may be preserved for a few days without hardening by immersing them in glycerin one part, water three parts. This keeps the material soft and pliable, and also acts as a macerating agent, rendering the tissues very soft so that they may be teased apart or even shaken apart with little difficulty. Hence the material must not be allowed to remain too long in the solution or it will be worthless.

The preparation of insects for microscopic study takes several forms. Small species may be mounted whole, in the round, or flat. They may be sectioned to show the internal arrangement of organs, or they may be dissected to show individual organs. The student will gain valuable experience in mounting by preparing a set of slides of various small species, mounting them flat as described below.

To mount an insect whole, flat, select some of the small

flat forms such as gnats, small ants, small spiders or small dorsiventrally flattened species of beetles, such as the Cucujidae genus, a good example of which is shown in Fig. 46. This is *Sylvanus surinamensis,* a small brown beetle frequently found in corn meal, flour and other prepared cereals. As already stated, the exoskeleton of adult insects is composed of chitin. In many species this is strongly pigmented and must be bleached before mounting, or it will not transmit the light needed to observe details. The black ant is a case in point. To bleach out the color, a weak solution of calcium hypochlorite in alcohol is used. This chemical is readily available in the commercial product Chlorox. To 10 ccm of 70% alcohol add three to four drops of Chlorox and immerse the specimens. Bleaching will require from a few days to as many weeks depending upon the size, permeability and depth of coloration of the insect. If bleaching is not complete at the end of two days pour away the solution and replace it with fresh. Repeat at two day intervals until bleached. Do not use a stronger bleaching solution than that recommended in an effort to hasten the process, for the ligaments that hold the various parts of the body together may be destroyed and the specimen will fall apart.

When bleaching is complete, pour away the solution and wash the material several times with alcohol to free it of any remaining chlorine. Next place the specimen under the dissecting microscope and spread the legs and antennae. This may be somewhat difficult, for the alcohol sometimes renders the subject too hard to spread. In this case do not attempt to force the parts into place, for this will result only in ruining the specimen. If any difficulty is experienced, if the parts do not remain as they are placed but snap back into their former position, place

the specimen in warm water (40° C.), for a short time, when the appendages should be sufficiently softened to be handled easily. This measure is to be adopted only in the most refractory cases, for the water immersion will destroy part of the work that has already been done. The soaking in alcohol has started the process of dehydration; some of the water has been extracted, and if the specimen is placed in water to soften it, this water will have to be again extracted before the subject may be mounted.

When the legs, antennae and wings have been spread to your satisfaction, lay a narrow strip of paper on each side of the specimen and press another slide down on it, holding the two slides together with a wrapping of thread, or clipping them together with a wooden spring-type clothespin. If the specimen was taken from 70% alcohol it must now be placed in 95% alcohol by immersing the two slides in the liquid. Leave for at least twenty-four hours. If the specimen was softened for spreading by soaking in water it must be placed in 70% alcohol for twenty-four hours, followed by 95% alcohol for the same length of time. The steps in the process are then the same for material prepared in either way. From 95% alcohol transfer the two slides with the specimen between them to absolute alcohol for a few hours, separating the slides slightly by releasing the thread that holds them together. Then separate the slides, remove the specimen with a camels-hair brush and allow it to remain in absolute alcohol for several hours longer. Prolonged immersion in absolute alcohol will not do the slightest harm, but if the specimen does not remain in it long enough to become completely dehydrated it will be impossible to clear it.

When you are satisfied that the material is perfectly free from water it must be rendered transparent. This is

accomplished by using a chemical that is miscible with both alcohol and Canada balsam. Xylol fulfils these conditions but has the disadvantage of hardening too much, thus rendering the tissues brittle. Furthermore, it will not clear specimens that contain the least trace of water. To overcome these disadvantages a mixture of xylol and beechwood creosote, equal parts of each, is used. This mixture clears the material perfectly, absorbs small traces of water and does not make the structures too brittle.

To use the creosote-xylol clearer, pour off the last wash of absolute alcohol and quickly add a mixture of equal parts creosote-xylol and absolute alcohol. Leave the material in this overnight, keeping the vessel well covered. Now replace the creosote-xylol-alcohol with creosote-xylol and leave the material until perfectly clear. When it seems to be cleared, replace the old solution with new and leave for several hours more, or until needed. If whitish opaque spots appear in the material it was not completely dehydrated and will have to be returned to absolute alcohol and again cleared until the spots disappear. In place of creosote-xylol, synthetic wintergreen oil (methyl salicylate) may be used, although it presents no advantages.

When the material has been cleared it is ready to be mounted on the microscope slide in Canada balsam, generally referred to simply as balsam. Hard, robust types such as ants, beetles and the legs of chitinous species may be transferred directly to thick balsam without any intermediate treatment. Soft-bodied specimens such as aphids, small larvae and the like must be slowly infiltrated with a gradually strengthened solution of balsam to prevent destructive osmotic currents from being set up by the difference in density of the clearer and the balsam.

The gradual introduction of balsam can best be effected by placing the material in a circular dish and covering it with creosote-xylol to a depth of about a quarter of an inch. Now fold a disc of coarse filter paper into the usual cone shape, adjusting the last fold so that a shallow cone is formed, the edges of which rest on the rim of the dish and the apex dips only slightly below the surface of the liquid but does not touch the material. Into this cone are placed a few drops of thick balsam which will be slowly dissolved by the xylol. More balsam is added from time to time until the liquid in the dish assumes the consistency of thin syrup. The filter paper is then removed and the mixture allowed to concentrate by evaporation until it is almost as thick as the regular mounting balsam, the vessel being meanwhile covered loosely with a glass plate to prevent the entrance of dust.

Thus prepared, the specimens are ready to be placed upon the slide. If the material has been carefully carried through the above stages it should be in excellent condition for critical study under the microscope. Considerable time and material have been used in bringing the work to this stage, and having arrived, it deserves proper mounting to display every characteristic and preserve it for future study. Work slowly, think out each step of the process and be sure you know what to do and how to do it before proceeding. Attention to a few details will insure perfect mounts which will retain their beauty indefinitely. Be careful to place the specimens in orderly arrangement if they are large enough to be handled individually and if more than one is included under one cover glass. If only one is used see that the appendages are arranged in life-like positions. This can be done best under the dissecting microscope with the aid of fine needles,

being careful not to pull off any of the appendages. Try to estimate the amount of balsam necessary to just fill the area of the cover glass and run out to the rim in a neat beveled edge. This will not be easy at first, but after some practice will not be difficult. Be sure to support the cover glass over delicate specimens so they are not crushed. This may be done in any one of several ways. Thin glass rods made by drawing tubing out in the flame make excellent supports for thick objects such as the larger Crustacea, spiders, ants, flies, small beetles, etc. Thinner specimens may be safely mounted by laying a piece of horse hair on either side, or cells may be made with shellac. Very small or thin objects require no cover glass support. They are placed directly on the slide, a small quantity of balsam dropped on them and the cover glass lowered carefully to prevent inclusion of air bubbles, which are very difficult to remove once they have formed.

A very useful accessory for mounting is made by ruling a rectangle the size of the slide on a piece of paper and drawing diagonal lines from each corner to locate the center. If a number of small specimens such as Paramecia are to be mounted they may be placed in a small heap in the center of the slide. A drop of balsam is then placed on them and the cover lowered into place. As many individuals of small species should be mounted as the supply of material will permit, for in this way a possible diversity of form will be presented. If deliberate arrangement is possible some individuals should be placed dorsal side up and others ventral side up, with a few showing the lateral aspect. This arrangement will permit critical study of every detail. Make every effort to prevent clumping of the material, for overlapping specimens will not reveal their characteristics. Extremely minute objects are best

mounted by placing a very small drop of balsam on a slide. Pick up the material with a fine pipette or a thin wire bent into a tiny loop and place in the drop of balsam. Allow this to harden overnight in a place free from dust. Next day more balsam, the supports and cover glass may be added without disturbing the mount.

Choice of a cover glass will depend upon the specimens. Small objects are best mounted under a circular cover, while larger ones or a large number of small individuals require a square glass. Select a size that will cover the specimen and allow 2 mm to 3 mm overlap at each side. Very small or thin objects to be examined under high powers or with immersion objectives should be mounted under No. 1 covers. Larger specimens for examination with medium and low powers may be satisfactorily mounted under the thicker No. 2 covers, which are less likely to be broken in handling the finished slides.

Placing the cover glass so as to avoid air bubbles is sometimes a bit difficult. A few general hints may be of assistance. Use a sufficient quantity of balsam to completely fill the cover glass area and extend beyond the edge in a rim from 0.5 to 1 mm wide all around. Balsam shrinks somewhat in drying and if the excess is not sufficient it may shrink back and expose the edge of the cover and even draw in air bubbles that might mar the preparation. When a sufficient quantity of balsam has been placed on the slide pick up the cleaned cover glass in a pair of fine-pointed forceps, warm it gently and quickly move it over the preparation on the slide. Lower the edge opposite to that held by the forceps until it touches the balsam and rests on the slide or supports, then slowly let down the held edge so that the balsam runs along the cover, but without disturbing the material. Release the glass and

allow its weight to press down upon the balsam. Now gently warm the slide on a warming stage or over a small flame until the balsam spreads slightly beyond the edge of the cover. Press lightly on the center of the cover with the blunt end of some instrument, such as the handle of a dissecting needle. If the quantity of balsam is insufficient to completely fill the cover glass a small amount may be added with a needle applied to the rim. If there is an excess beyond that required for a neat rim it must be carefully scraped away without disturbing the cover.

Fig. 48. Completed slide.

The slide is now ready to be labeled. The label is a small sheet of gummed paper attached to the slide. On this is recorded all pertinent information, including the name of the object, the date it was received and mounted, the fixing solution and the stain used, and the number of the slide. This number should be recorded in a note book together with the data contained on the label, and any other data that may be of interest. Such data might consist of the exposure time, the light and magnifying power used, the filter, if any, and the type of film if the subject was photographed. The methodical recording of such data is of inestimable interest and value as a working basis for subsequent photographs.

Chapter IX

Preparing Vegetable Specimens

Rich as the vegetable kingdom is in material for the microscopist, the student will find excellent exercise in the examination of plant stems for his first adventure into vegetable microscopy. These important structures of plant life are interesting subjects and will afford endless hours of instructive study if pursued with the assistance of a good book on botany. While a detailed discussion of stem features is impossible here, we can at least make a few introductory remarks to enable the student to appreciate some of the things he sees when he places a stem section under the microscope.

The stem of a plant, and this includes all stems from the short stubby stems of turnip to the enormous trunks of our giant redwoods, serves two primary purposes. It supports the foliage, holding the leaves up into the light and air, and serves as a food canal, carrying the moisture and food that is absorbed by the roots up into the aerial parts of the plant to nourish them. A secondary function of the stem is as a means of the sort of reproduction is effected by branching and budding.

The botanist recognizes two well defined groups of stems, known as endogenous, or inside growing, and exogenous, or outside growing. These terms refer to the arrangement of the structural elements. Dicotyledonous stems or dicots are examples of the exogenous type, while monocotyledonous stems or monocots are of the endogenous type. The stems in both systems are composed of hard, woody bundles, called fibro-vascular bundles.

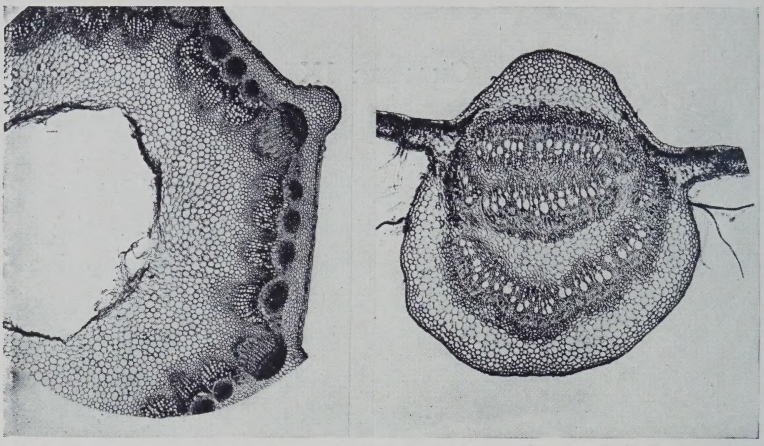


Fig. 49. Cross-section of tansy stem. X20

Fig. 50. Cross-section of oak leaf at midrib. X32

Slides and photographs by Irving L. Shaw

These are surrounded by a mass of more or less soft material, over which is the bark or protective coating. The arrangement of the bundles in the two systems is quite different. In the exogenous type the bundles are nearly always arranged in a circle near the outside of the stem, and the strength of the stem depends upon their compactness. Such stems will increase in diameter as long as the plant lives. This increase is the result of growth in the layer of cells lying just under the epidermis or outside layer. These growing cells form the cambium layer, and by their growth each year give rise to the annual rings that may readily be seen in a cross-section of a tree trunk.

Endogenous stems are again divided into two groups, herbaceous and woody. Herbaceous stems consist of a mass of soft, pithy cellulose surrounded by a thin hard layer of tissue containing the cambium and the epider-

mis. Scattered irregularly through the soft core are the fibro-vascular bundles, or simply bundles as they are commonly called. All of the grass stems are endogenous, as are those of rushes, iris and numerous other plants. Corn, which is a coarse grass, is a good example of an endogenous herbaceous monocot. There is another type of endogenous stem that is much firmer in texture, although of the same general character. This is in the form of a cylinder in which the bundles have been compressed into a thin ring, making the stem tubular and of great strength. The various grain stems and most common grasses are examples of this type. Soft juicy stems in which the bundles are small as compared with the surrounding material are called herbaceous, while those in which the bundles constitute the greater part of the stem substance are called woody. Most of the strictly woody stems are exogenous and show annual rings.

Some plants have underground stems that are frequently mistaken for roots. The May apple (*Podophyllum peltatum*) is a good example. The underground stem may be distinguished from the roots by the more or less regular division of the stem into nodes and internodes. Branching from these underground stems always occurs at the nodes, and is therefore regular. Roots are without these nodal divisions, hence the branching is very irregular. The common white potato is a good example of an underground stem of the tuber type. Leaf scars are present in this type of stem in the indentations we call eyes. Each of these eyes is a bud and a potential potato stalk.

Rhizomes, or as they are more commonly called, bulbs, are another form of underground stem. Cut a median longitudinal section of a tulip bulb and the laminated

structure will at once be evident. These laminae are in reality modified leaves that surround the embryo. When the plant grows and before it has developed to the point where it can manufacture its own food, it draws its nourishment from these modified leaves. That is why many tulips received from greenhouses as full grown plants die in a short time. Growth has been so rapidly forced in soil of low fertility that almost the entire food supply has been consumed in growing leaves and flowers. When the small remaining amount of available food has been used the plant dies and the bulb becomes a shriveled mass.

This matter of food storage for the young plant is quite important. When the seed germinates it sends out the root hypocotyl first in order that the process of supplying nourishment and water may begin at once. Then the plumule or young leaves start to grow. This growth is very rapid, so rapid that the young roots are inadequate to supply the food needed and the young plant would starve, had nature not provided a source of food to keep the plant alive until the roots begin to function. This food supply is contained in the seed itself, and consists largely of starch and a small amount of minerals. Through the actions of enzymes produced within the seed cells this food supply is made soluble, or otherwise modified so that it is available for the young sprout until its own food manufacturing process begins.

Now let us return to the preparation of vegetable material. In general, preparation for microscopical examination does not differ from the schedules already outlined for animal material. Some authors recommend the use of celloidin for imbedding because of the high water con-

tent of all vegetable tissue. Chamberlain, however, uses paraffin with complete success in his work in plant histology. There is therefore no reason why the student cannot secure satisfactory results with the same imbedding medium. The writer has been successful with subjects such as ovaries of flowers, in which cut portions of embryo seeds left unattached in the section were mounted on the slide in the same relative positions they occupied in the growing capsule.

The primary requisite for success in preparing vegetable specimens lies in strict adherence to proper practice in every detail of the various operations. Perhaps a summation of the principal steps will not be amiss.

1. Fix and harden all tissues thoroughly. Bouin's fluids are as satisfactory as any fixative. The alcoholic solution penetrates rapidly, fixing rose ovaries in one hour if sections are required quickly. The aqueous solution is slower but just as thorough if given enough time to act. Be sure to cut off one end of the ovary to insure complete penetration of the fixing fluid. Bouin's leaves the material in excellent condition for staining with any stain the student is likely to use. Being a picric acid mixture, Bouin's must be washed out of the fixed material with 70% alcohol, and *never with water.*

2. Dehydrate by passing the material from 70% alcohol to 80%, then 95%, and finally complete the process in absolute alcohol. Allow plenty of time for complete extraction of the last traces of water, and change the absolute alcohol at least once, allowing an equal period of immersion in each change.

3. The transfer to xylol must be gradual. From absolute alcohol transfer the material to:

A absolute alcohol, 75%, xylol 25%, then
B absolute alcohol, 50%, xylol 50%, then
C absolute alcohol, 25%, xylol 75%, then
D pure xylol

When the material appears clear and transparent throughout, pour off the xylol and add pure toluol, allowing it to remain in this for several hours.

4. Now pour off the toluol, add a quantity of fresh solution and begin infiltrating with hard paraffin. Add the paraffin gradually until the toluol is saturated at room temperature. This is especially important with vegetable materials since these contain much included air in minute bubbles that must be displaced with paraffin, else the material will break down in sectioning.

When the toluol is saturated, transfer the container to the paraffin oven at a temperature of 35° to 37°C. and allow the slushy mixture of paraffin and toluol to clear by complete solution of the paraffin. Then add more paraffin until the solvent is saturated at the oven temperature. Let it stand in the warm oven overnight, then pour off half the mixture and make up to its former volume with finely shaved paraffin. Raise the oven temperature to a point just above the melting point of the paraffin, as determined by test. Keep at this temperature for twenty-four hours. Do not allow the temperature to rise. At the end of this time pour off all the toluol-paraffin mixture and add fresh melted paraffin. Leave it in the oven until all the residual toluol has evaporated.

5. Remove the container from the oven, place the material in imbedding trays and add melted paraffin. Solidify the paraffin in cold water.

All of the steps included in the above summary have been described in detail in Chapter V. Several modifica-

tions have been introduced to adapt the process to vegetable materials, but in the main the operations are identical. For instructions covering the cutting of sections see Chapter V.

6. Stretch the sections on albumenized slides.
7. Remove the paraffin.
8. Coat with celloidin.
9. Downgrade to water.
10. Stain.
11. Upgrade to absolute alcohol.
12. Counterstain.
13. Recoat with celloidin.
14. Clear and mount in balsam.

CONIFEROUS LEAVES — The leaves or needles of coniferous trees are interesting botanical subjects for study. To prepare sections take a bundle of needles, tie them together with a strand of silk, imbed the bundle in paraffin and cut transverse and longitudinal sections.

POLLENS — The study of flower pollens embraces such a vast amount of material that the student could devote years of study to this branch alone. Pollen may be prepared either as opaque or transparent material. Usually both methods are used, frequently on the same slide.

Opaque mounts are made by spinning a shellac cell on the slide, of slightly greater depth than the thickness of the pollen grains. A disc of dead black, matte-surface paper is then pasted to the inside of the cell with thin shellac or celloidin. When this is perfectly dry, coat the upper surface of the paper with gum water and allow it to dry. Breathe on the paper and quickly dust the dried pollen grains on it. The breath will moisten the gum enough to make the grains adhere. Set the slide in the desiccator to dry thoroughly, then spin a ring of shellac

on the cell and imbed the cover glass, finishing with another ring of shellac.

Transparent mounts of pollen are somewhat difficult to make because of the tenacity with which the grains retain air bubbles. Many species of pollen are covered with fine hairs, the interstices between them holding the air imprisoned. Place the dried pollen in rectified spirits of turpentine for several days, then transfer it to 70% alcohol to remove the turpentine. Replace the 70% alcohol with 95% alcohol and allow to remain several days longer. Transfer to absolute alcohol until completely dehydrated, clear in toluol and mount in balsam, supporting the cover glass with glass rods.

To mount pollen in balsam a slight variation must be made in the usual technique. Place a drop of thin balsam on the slide and spread it with a needle until it covers an area equal to that of the cover glass, being sure to break any bubbles that form. Drop the cleared pollen grains into the balsam and set in the desiccator until dry. Now add another drop of balsam and the glass rods, warm a cover glass over the spirit lamp and lower it on the slide carefully to avoid bubbles.

Deeply pigmented pollens like that from the tulip must be bleached in chlorinated alcohol as described in Chapter VIII. Colorless varieties may be stained to increase visibility. The best stains for this purpose are those anilines soluble in alcohol, such as eosin, fuchsin, safranin, methyl violet, malachite green, methyl green, etc. Use a rather strong solution, say 2–3% in 95% alcohol and stain until the desired depth of color is attained. Pour off the stain, rinse in absolute alcohol, clear in beechwood creosote and mount in balsam. If the staining has been too deep it may be reduced by immersion in alcohol.

GERMINATING SEEDS — The dried seeds of cereals (wheat, oats, barley, buckwheat, etc.) are soaked in water for a few hours until they resume their natural shape as nearly as possible. Then place a disc of blotting paper in a Petri dish or saucer, moisten it and spread a layer of swelled seeds on the paper. Cover this with another disc of moist blotting paper and place in the warm oven (about 25°C.) until the embryo begins to germinate and sprout. Germination can be arrested at any desired point by throwing the seeds in alcoholic Bouin's fluid. Allow to fix thoroughly, then dehydrate, infiltrate and imbed in paraffin and cut longitudinal sections.

Many vegetable materials, especially stems, may be cut in the well microtome without imbedding. Soak the stem in water until soft and then cut sections 20 to 30 μ thick. As the sections are cut place them in 70% alcohol for about ten minutes, then transfer them to 95% alcohol and allow them to remain twenty to thirty minutes. Now pour off the alcohol and replace with a 1% solution of safranin in 50% alcohol, allowing this to act about twenty-four hours. Pour off the stain (which may be used repeatedly) and replace with 50% alcohol to wash out the excess color. Lignified structures retain the stain more tenaceously than cellulose walls, which affords an opportunity to differentiate between the two. Destain until most but not all the color has been extracted from the cellulose walls and the lignified walls still show a strong, brilliant color.

If the destaining is very slow it may be accelerated by adding a small drop of HCl to the washing alcohol. Proper differentiation without acid should be complete in from five to fifteen minutes. A longer period indicates acid treatment.

Pour off the washing alcohol and wash the sections in tap water, being especially thorough if acid has been used for differentiating. The least trace of acid left in the sections will cause the stain to fade in a short time.

Now stain in Delafield's haematoxylin for ten to fifteen minutes. This will stain the cellulose walls but will have little or no effect on the lignified structures. Wash the sections in tap water for several minutes or until the original red color is replaced by a rich purple. If the cellulose walls show only a faint color replace in the stain for a longer period. When the color is a deep purple or nearly black, destain in acidulated water, using only the smallest amount of HCl and mixing it well with the water before allowing it to touch the sections. As soon as a reddish color appears in the sections, which may take place in from four to five seconds, pour off the acid water and replace with tap water. Wash well to free from acid. This acid treatment differentiates the stain by washing it out of the lignified walls more readily than from the cellulose walls, at the same time dissolving the precipitates that frequently follow staining with Delafield's. The remaining safranin is also washed out in this treatment, which is the reason for allowing part of it to remain from the first washing. If this were not done the stain would be weak in the lignified walls.

Run the stained sections up through the ascending series to absolute alcohol, clear in toluol or clove oil and mount in balsam.

If sections are to be mounted in glycerin jelly they may be mounted directly from the last wash water, since they are not dehydrated for glycerin mounting.

Clove oil may work better for clearing than toluol or xylol if the dehydrating absolute alcohol is not quite

100%. Clove oil will clear from alcohol of 99.5% or even 99%, so that if the strength of the alcohol is the least bit questionable it is better to be safe and use clove oil. The sections should then be placed in xylol for a short time to facilitate hardening, since material cleared in clove oil hardens very slowly in balsam.

The following schedule of the foregoing steps is given as a tentative guide for staining.

1. Sections in 95% alcohol.
2. Safranin, 12 to 24 hours.
3. 50% alcohol until color is right, generally 2 to 10 minutes. Acid alcohol may be used if needed.
4. Water, running for several minutes, or 5 minutes in water that is changed frequently.
5. Delafield's haematoxylin, 3 to 30 minutes.
6. Water, running, 5 to 10 minutes.
7. Water, slightly acidulated, 5 to 10 seconds.
8. Water, 20 to 30 minutes to wash out acid.
9. 50% alcohol, 1 minute.
10. 95% alcohol, 1 minute.
11. Absolute alcohol, 5 minutes.
12. Toluol, 1 to 5 minutes.
13. Balsam.

If clove oil is used for clearing, finish as follows:

12. Clove oil, 2 to 5 minutes.
13. Xylol, 1 to 5 minutes.

The following schedule makes use of malachite green and Congo red. Material is treated with 95% alcohol, then passed down to water and stained as follows:

1. 3% aqueous solution of malachite green, 6 hours or more.
2. Wash in water.
3. Congo red, 1% aqueous solution, 15 minutes.

4. Wash in water.

5. Rinse in 80% alcohol. As soon as the green color appears through the red, transfer quickly to,

6. Absolute alcohol.

7. Toluol.

8. Balsam.

Other stain combinations that are easy to use are iodine green and acid fuchsin; methyl green and acid fuchsin; safranin and gentian violet. The schedules given above may be followed with any of these combinations, with slight modifications as indicated by the material in process.

Chapter X

The Polarizing Microscope

No discussion of general microscopy would be complete without some reference to the polarizing microscope. Aside from the gorgeous colors produced by many materials in polarized light, the polariscope is of immense value in the study of mineralogy. No petrologist would ever think of studying a new mineral or crystal without using the polarizing microscope. Many minerals, because of their atomic structure, display the phenomenon known as optical activity. This means that the material takes on a different aspect in polarized light from that presented in unpolarized light. The number of optically active materials is legion. The characteristics of many of these in polarized light are known and afford an excellent and infallible means of identification, even when mixed with other materials. Others are still waiting for someone with enough time to investigate them, affording the student another opportunity for original research.

The polarizing microscope, or polarizer, as it is generally called, is almost like any other microscope. The differences lie in the addition of two prisms of calcite (described in Chapter XI) known as Nicol prisms, and a rotating stage. For student use the rotating stage is not absolutely necessary, but the nicols are. There are two of these, one called the polarizer, the other called the analyzer. The polarizer is located under the stage so that the light is acted upon before it reaches the slide. The analyzer is located between the slide and the eye of the ob-

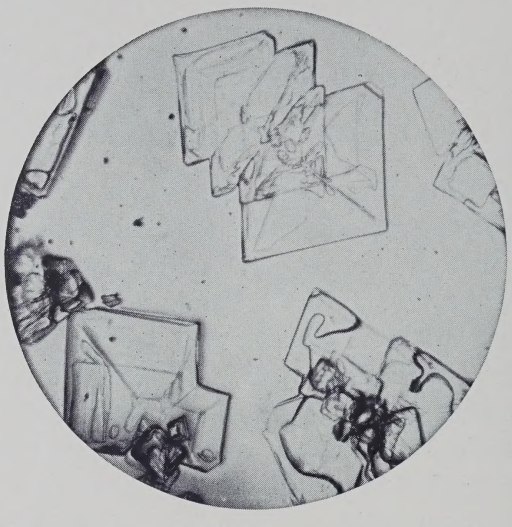


Fig. 51. Crystals of potassium chlorate photographed in ordinary light. Crystals grown in gelatine. Mounted in balsam.

server, usually in the draw tube of the microscope. One (or both) of the prisms must be so mounted that it can be rotated on its principal axis. This rotation makes possible alteration in the plane of rotation of the light so that this plane may be made parallel with or at right angles to the plane of vibration of the second prism. Thus, when the plane of vibration of the two nicols is in the same direction we say the nicols are parallel; when the plane of vibration of the one nicol is at right angles to the plane of vibration of the other we say the nicols are crossed. As the planes of vibration of the two nicols progress by rotation from parallel to crossed the field gradually darkens until, when the nicols are completely crossed, or exactly at right angles to one another, the field is quite dark. This is called the point of extinction.

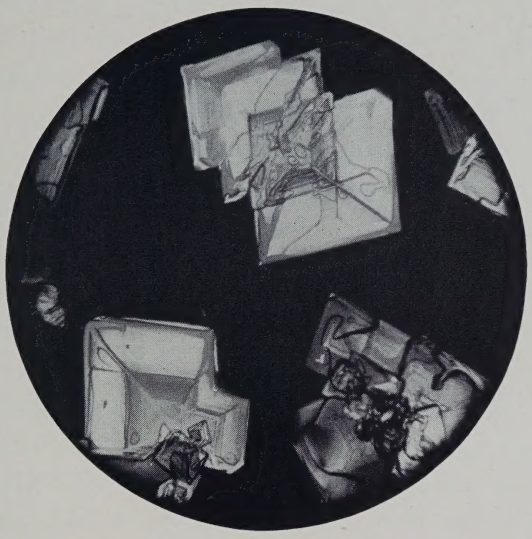


Fig. 52. The same crystals of potassium chlorate photographed in polarized light, crossed nicols.

While we cannot enter into a detailed discussion of the theory of polarized light, it may help the student if we explain just a little of that theory. Polarized light, it must be remembered, is just ordinary light. The difference lies entirely in the manner of vibration. In ordinary light, either natural or artificial, vibration of the ether particles that effect the transmission of light waves is in every conceivable direction across the light path. Now, when the nicol prism is introduced into this light path it acts as an optical grating altering that heterogeneous vibration and making the light vibrate in one single plane. Practically all of the incident light is transmitted but its many planes of vibration have been transformed into one constant plane. If another nicol is now allowed

to intercept the path of light from the first nicol and this second nicol is arranged so that the direction in which it permits ether vibrations to go is at right angles to the plane of vibration of the incident light, it stops the incident light entirely, thus acting as an analyzer.

If the student is interested in a more detailed account of the theory of polarized light he is referred to one of the texts mentioned in the bibliography. For our purpose the above brief description must suffice.

Most organic and many inorganic substances are optically active and will respond to polarized light, showing colors or interference figures. Aided by the home-made polarizer described in Chapter XI the student can enjoy the study of materials in polarized light. The following short list of polarizer objects and their method of preparation is included in the hope that they may afford a starting point for further investigation in this fascinating field.

Crystals of chemical compounds are great favorites. These may be prepared in several ways and will vary with each mode of preparation.

Method No. 1. The usual method of preparing crystals is to make a strong solution of the salt in distilled water. Filter the solution and place a drop on a slide. Several slides may be prepared, drying some naturally, protected from dust, evaporating others over the spirit lamp, and drying the rest in the desiccator. When thoroughly dry add a drop of balsam, except as noted later, and a cover glass supported to prevent crushing the crystals.

Method No. 2. Make a saturated solution of the salt in distilled water. Add a small quantity of gum acacia or gelatine, enough to make about a 5% solution, and filter. Place a drop on the slide and set in the desiccator to dry. Mount in balsam, except as noted.

Method No. 3. Place a small piece of the dry salt on a slide and lay a cover glass over it. Warm gently over the spirit lamp until the substance melts, press the glass down on the slide with a blunt instrument and allow to cool.

Method No. 4. Crystals of many salts can be produced instantly by pouring some of the saturated aqueous solution into alcohol, when the chemical is not soluble in alcohol. Use 95% alcohol and change it once. Then pour off the 95% solution and replace with absolute alcohol. Change this once to completely dehydrate the crystals. Mount in balsam except as noted.

Note. Some crystals, especially those of organic compounds, are soluble in the solvent used to dissolve balsam. These must be mounted in castor oil and given a protecting ring of shellac.

To mount in castor oil the preparation is made on a cover glass instead of on the slide. Dehydrate the crystal either in alcohol or in the desiccator. Spin a ring of shellac or gold size on the slide and allow to dry. When dry add another ring, building up the depth of the cell thus formed until it slightly exceeds the height of the cover glass preparation. When the cell is nearly dry fill it with castor oil, take the preparation in the fine forceps and bring it, crystal down, over the cell. Lower the cover glass in place gently to exclude air bubbles, then press the cover on to imbed its edge in the shellac. Set aside to dry. Clean off excess oil with xylol and spin a finishing ring of shellac on the edge of the cover.

Crystals may be mounted dry in the same sort of cell. All operations are the same except that no oil is used in the cell. Cells for deep mounts may be made as described for mounting insects in Chapter VIII.

Slides and cover glasses that are used for crystal growth

must be absolutely clean, free from any trace of grease or dust. Any foreign matter on the slide or cover glass will interrupt the formation of the crystals, preventing normal development.

An interesting experiment in crystal formation may be conducted with a strong, but not saturated, solution of a salt that is placed on the slide and allowed to dry in the air of a room in which people are moving about. The reason for using a strong rather than a saturated solution is that crystallization might begin too soon. Place a drop of the solution on a clean slide and set it where dust particles will fall into it from the air. Presently, as the water evaporates and the liquid reaches the saturation point, crystals will form about each dust particle as a nucleus.

Crystals of Metallic Silver — While on the subject of crystals let us examine a method of making a very beautiful slide of metallic silver crystals. These crystals do not polarize, but are mounted in an opaque cell on the slide. Place a perfectly clean slide in the turn table and spin a shellac cell, making it of fair depth. While the cell is drying make a 1% solution of silver nitrate in distilled water. When the cell is perfectly dry place a drop of the silver solution in the center and drop a few fragments of copper, made by filing a copper wire, into the solution. Set away in a place free from dust and in a short time beautiful dendritic crystals of metallic silver will form around the bits of copper dust. Leave until perfectly dry, spin a ring of shellac on the cell and imbed the cover glass. Paint the back of the slide with India ink or opaque black show-card color to make a black background.

Starches — All starches polarize beautifully, either with or without the mica plate. Without the plate they

show the characteristic cross in black, while with the plate the grains are beautifully colored, the cross still remaining. The shape and size of grains of starch varies with each vegetable substance from which it was taken. It is insoluble in cold water, hence is easy to procure in a free, unadulterated state from grains and vegetables. To obtain starch grains from tuberous-rooted vegetables such as potatoes, sweet potatoes, yams, etc. simply scrape the cut surface with a knife and place the scrapings in a test tube of distilled water. When a sufficient quantity has been collected shake it well and strain through linen fine enough to retain the large particles of cellular tissue but allow the starch to pass. Allow the starch to settle, which will take place in a few minutes, then decant the water and replace with clean water. Repeat the washing several times until the starch is perfectly clean. Preserve in distilled water.

Starch grains for the polarizer should be mounted in balsam for best results. When the starch is clean, prepare a slide with Mayer's albumen fixative (Chapter V). When nearly dry add a drop of water containing the starch. Spread evenly with a needle and allow to stand in a dust-free place until nearly dry. The film should be just moist, not wet enough to run when the slide is tilted. Plunge the slide in this condition into 70% alcohol to coagulate the albumen, then transfer to 95% alcohol and dehydrate in absolute alcohol. Clear in toluol until transparent and mount in balsam.

Dried specimens such as cereal grains must be soaked in cold water until soft enough to scrape, then treated as above. Small seeds should be placed in a mortar with water and broken into small bits. Prolonged maceration of the seeds in cold water will eventually break up the

cells and liberate the starch grains, when they are treated as above.

STARCH IN SITU — To show the arrangement of starch grains in their natural positions in the cells the material is first cut into small pieces and dehydrated by passing up the ascending series of alcohols to absolute alcohol. Since the character of starch is altered by heat, starch-bearing materials cannot be imbedded in paraffin for sectioning. To cut sections make a cylinder of carrot to fit the well microtome. Split this through the center longitudinally and hollow out each half so that when they are placed together the object to be cut will be gripped tightly. Force into the microtome and cut fairly thick sections. As the sections are cut place them in absolute alcohol. Add to this alcohol enough of a 2% solution of methyl violet in absolute alcohol to color it strongly. Prepare the slide beforehand by applying a very thin film of diluted balsam, and allow to dry. While the balsam is drying the stained section should be clearing in toluol. When it is perfectly clear place it on the prepared slide with a section lifter. The toluol remaining in the section will soften the balsam enough to make the section adhere tightly. Add a drop of balsam and a cover glass, supported on glass rods.

Sections of watery materials may be mounted in glycerin jelly if the student does not wish to go through the more involved processes required for balsam mounts, although glycerin preparations are not as clear as balsam preparations. Pass the sections down the alcohol series to water, stain in aqueous methyl violet or malachite green, wash away excess stain with water and mount in glycerin jelly. (Chapter VIII.)

RAPHIDES — These crystals of calcium oxalate are pres-

ent in a large number of vegetables and plants. Cacti are very prolific, as are the cuticles of onions, garlic, hyacinth and lily. Rhubarb is a good source of raphides, which may be procured by squeezing the juice from a cut end of the stem onto the slide. The name is from the Latin *raphis,* meaning a needle, and is suggested by the form many of them present. Some are long and slender, others are set in groups of rays like a rosette, while still others are short and stout. They vary in size from extremely small ones to some as large as 1/25 of an inch. They give brilliant colors with the polarizer.

Raphides are insoluble in water, hence can be procured by maceration in water until the tissue falls apart. The larger pieces are picked out and the material washed repeatedly in water. Between each washing the raphides are allowed to settle. Pipette off the supernatent liquid until no plant tissue remains. Dehydrate in absolute alcohol, clear in toluol and mount in balsam, supporting the cover.

Silicious Skeletons — Many of the grasses, canes and cereal grain stems have a skeleton or cuticle of silicate which makes a splendid polarizer object. The material is first dried, either by heat or alcohol, and then immersed in strong nitric acid and boiled. An effervescence will take place as the tissue is destroyed, and when this stops more acid must be added. Boiling and the addition of fresh acid must continue as long as there is any effervescence. Then replace the last acid with fresh and allow to macerate until all tissue is dissolved. Wash in distilled water until free of acid, using litmus paper as an indicator, dehydrate in alcohol, clear in toluol and mount in balsam.

Leaf Cuticles — The thin outer coatings of leaves are

very interesting subjects when separated from the leaf and examined by polarized light. Many leaves are amenable to simple maceration in water until rotten, when the cuticle can be stripped off in fairly large pieces. Other leaves of hard texture like the rhododendron, azalea, laurel, etc. should be cut into pieces about a quarter of an inch square, placed in a test tube with strong nitric acid and heated until a separation of the cuticle begins as indicated by the formation of blisters on the leaves. Throw the contents of the test tube into a large volume of clear water as soon as this occurs, to arrest the action of the acid, before the cuticle dissolves.

Separate the cuticles with dissecting needles, wash them well in distilled water to free from acid, pass through 50%, 95% and absolute alcohol, clear in toluol and mount in balsam. The cuticles may be stained in malachite green by adding a few drops of an alcoholic solution to the absolute alcohol.

Leaf Scales and Leaf Hairs — Leaf hairs exhibit an enormous variety of forms and polarize beautifully. Sections of leaves containing hairs may be made as instructed in Chapter V, if it is desired to show them attached to the leaf. While this is instructive, the true arrangement of the hairs is not always revealed because the paraffin imbedding flattens them. They may be procured as isolated objects by hardening the leaf in alcohol and then cutting off the hairs close to the leaf with a sharp scalpel or razor. Collect the hairs on a filter and handle them through dehydrating and clearing as described in Chapter V. The best results are secured when hairs are arranged in order on the slide. It is tedious work but well worth the effort. Prepare the slide as described for mounting starches. Then, using the fine-pointed red sable brush to handle it,

place each hair separately under the dissecting microscope. They may be arranged radially, as the spokes of a wheel, in rows, or in crosses. The latter arrangement is especially interesting since it produces another color where the two hairs intersect.

Leaf scales are procured by scraping the leaf surface. These objects are very difficult to free of air, requiring prolonged immersion in the clearing fluid. Dehydrate in absolute alcohol, clear in toluol and mount in balsam.

TEXTILE FIBERS — The fibers of cotton, flax, silk and wool are all optically active. If the raw fibers are available they should be mounted individually as type specimens for comparison with test subjects. Dehydrate in absolute alcohol, clear in toluol or creosote-xylol and mount in balsam.

Samples of woven fabrics may be prepared in the same way and these afford especially beautiful polarizer objects.

RADULA OF SNAILS — Tongues of snails are excellent objects for dissection. If the specimen is large enough so that the tongue may be easily dissected out, do so, being sure the coiled up portion found inside the snail is removed with it. Place in 10% potassium hydroxide solution for a few days to destroy the soft parts. Wash well with distilled water and spread the tongue on a slide. Lay a strip of paper on either side of the object to prevent crushing it and cover with another slide. Tie the two slides together, dehydrate in absolute alcohol, then clear in creosote-xylol. When the object is cleared cut the string binding the slides and separate them. The tongue will probably adhere to one of the slides. Leave in creosote-xylol for a while longer, then transfer to a clean slide and mount in balsam.

HAIRS — The hairs of all animals provide splendid

polarizer objects, both in transverse and longitudinal sections. The usual method of procuring sections of hairs is that of shaving closely without the use of soap. While this works after a fashion, the sections are usually diagonal-transverse, since hairs do not grow perpendicular to the skin. A better method, in which exactly transverse and longitudinal sections can be had is as follows.

Take a bundle of hairs and tie them together with another hair. Make up a strong solution of gelatin by solution in water, liquifying it on the water bath. Immerse the bundle of hairs in the gelatin and leave until entirely impregnated. Remove on the point of a needle and expose to the air until the gelatin cools and becomes semi-solid. Push the mass off the needle into 70% alcohol and allow it to harden. Dehydrate in absolute alcohol, imbed in carrot and cut sections in the well microtome. The sections are then dehydrated, cleared in creosote-xylol and mounted in balsam.

The only objection to this method is the fact that the imbedding gelatin is included in the mount. By making paraffin sections this objection is overcome and the isolated sections of the hairs only are on the slide.

To make paraffin sections the bundle of hairs is prepared as above, dehydrated, cleared and infiltrated with paraffin exactly as described in Chapter V. All operations are carried out as described there, up to the point of dehydration in absolute alcohol. Since these sections are not being stained they are not passed down the graded alcohol series but are cleared in toluol directly from the absolute alcohol and are then mounted in balsam.

Horny Tissues — Structures that are composed of compressed cells, such as quills, horns, hoofs, claws and nails must be macerated in 30% solution of potassium hydrox-

ide until soft. The period of immersion will depend upon the degree of hardness of the material and the size of the pieces. When soft enough to cut, wash the material well in water to remove all trace of alkali and preserve in alcohol until needed. Hollow objects, like quills, small horns and small claws should be then dehydrated, infiltrated with and imbedded in hard paraffin for sectioning. Hoofs should be cut in carrot and the sections handled as loose sections, or they may be flattened on albumenized slides and handled like paraffin sections. Both transverse and longitudinal sections should be made to demonstrate the cell arrangement.

Chapter XI

Accessories

In the introduction that prefaced these chapters a statement was made that microscopy is not expensive. References to chemicals and equipment may raise doubts as to the veracity of that statement. Such doubts would be quite justified if the student based his conclusions on the cost of accessories as listed in the supply catalogs. Fortunately there is another course open to him, for nearly every piece of accessory equipment can be made at home, or substitutes can be provided at low cost. There are some items, such as dissecting knives, which, for the sake of comfort in working, should be purchased. As for chemicals, there are really only a few chemicals needed. Most of them are comparatively inexpensive and only small quantities of each are used. Therefore, the statement that microscopy is not expensive is true, as it is the purpose of this chapter to prove by discussing the equipment needed, the ways of making it at home and the substitutes for commercially manufactured items.

Dissecting Microscope — A dissecting microscope is quite essential for separating objects to secure parts to be studied. Fig. 53 opposite shows an inexpensive form of manufactured instrument. While such an instrument would be pleasant to work with, a satisfactory substitute can be made at a cost less than the cheapest manufactured product by supporting a lens in a holder made of stiff wire. The lens used has a magnifying power of 7.5 and costs seventy-five cents. The student who has facilities for doing the work can make a dissecting microscope like

the cheaper one illustrated, with very little trouble, using the lens mentioned above.

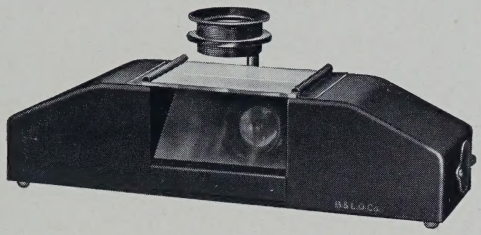


Fig. 53. Bausch & Lomb inexpensive Dissecting Microscope for student use. X7.5

Dissecting Needles — Dissecting needles were mentioned in Chapter VIII. The catalogs list these items in several styles, with bone, metal or wood handles and equipped with little chucks to hold the needles. These instruments look very professional and cost in proportion. Two are shown in the lower right corner of the illustration, next to the large forceps.

Satisfactory substitutes for commercial dissecting needles can be made at home for a few cents. After all, the handle is not important. All it is for is to hold the needle, and for this purpose wood is as good as any other material. Hammer shanks from pianos, which are smooth, round sticks of maple about three-sixteenths of an inch in diameter, are excellent for this purpose and may be procured from any piano repairman simply for the asking. Cut the ends square and bind one end to form a ferrule by wrapping it tightly with fine copper wire for about a quarter of an inch. The ends may be soldered to hold them in place. Now take the needles you propose to use, break or grind off the eye and grind the end to a point on a sharpening stone. Hold the needle in a

vise and drive the handle on it with light taps to a depth of about a quarter of an inch. Thin needles are not likely to split the wood if the handles are ferruled.

Handles for heavy needles such as are required for some dissections must be prepared for the needle by drilling or burning a small hole before the needle is driven home. These, too, should be ferruled since they are sometimes used under considerable pressure and might split. Lacking a small drill, burn the holes with a needle smaller than the one to be used.

The number and shape of dissecting needles is entirely up to the student. They are so inexpensive that a sufficient number should be provided to insure clean ones always being at hand ready for use in emergency. Most work may be done with a selection of four or five sets of two each. One set should have extremely fine points for the most delicate work, such as teasing apart single cells. For these use the finest sewing needles, or the finest grade of entomological pins. These, with the heads removed, make excellent needles. One set of two needles should have the points bent at right angles to the shank, while two should be straight. One pair of curved and one pair of straight needles in a heavier weight will be needed for coarser dissections that would destroy the delicate points of the fine needles. A third set of two straight and two curved heavy needles with blunt points should be provided for very coarse work such as tearing apart large pieces of tissue to be later teased with the finer needles. Always keep the points clean and sharp by grinding on a carborundum stone.

Dissecting Knives — In the upper right corner of the illustration (Fig. 54) are four of the Bard-Parker knives mentioned earlier. Directly under these is another style

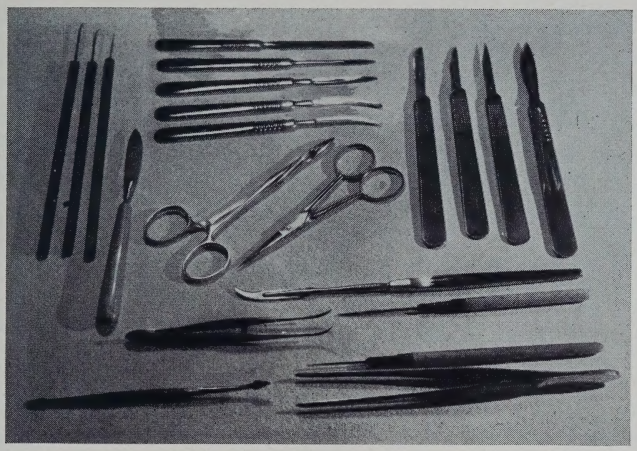


Fig. 54. Dissecting instruments.

made by the same manufacturer. These are the most useful dissecting knives to purchase, for reasons given in an earlier chapter. At the upper center is a group of four lancets and a bone saw. While these delicate instruments are very useful at times they are not absolutely necessary if the student has a Bard-Parker handle with the No. 11 and 12 blades.

FORCEPS — Three types of forceps are illustrated, one with fine curved tips for setting cover-glasses, picking up minute objects and for use in dissections. They may be procured in most drug stores at very small cost. The large forceps in the lower right corner is useful for taking specimens out of bottles and holding slides in the flame for warming, as well as in dissecting. The third forceps in the upper center is known as a suture forceps. It is used by surgeons to hold the suture needle when sewing wounds, but it finds many uses in dissection work. For example, when slitting a large piece of material or an entire animal to get at some internal organ, this instru-

ment is used to hold one side of the subject and keep it out of the way. The corrugated jaws afford a firm grip on slippery material, while the handles are equipped with a simple lock that keeps the jaws closed until deliberately opened.

Shears — Poor shears are worse than none at all. A pair of good, small shears may cost a dollar or more, but are worth every cent of it. The ordinary scissors sold by department stores for embroidery work are usually poor specimens. Go to a cutlery store and select a good pair of shears about four inches long. Be sure they have fine points that meet perfectly and cut cleanly to the extreme tip. This is important, as in dissections it is very frequently necessary to make short cuts that can be accomplished only with the very tips of the shears. Keep them sharp and clean and do not strain them by cutting hard or tough materials beyond their ability, for this distorts the points and destroys their fine adjustment. Manicure shears with fine, curved points are sometimes useful but not essential, for the lancet or scalpel may be pressed into service if it is necessary to reach a difficult place.

Section Lifter — Loose sections cut from bulk objects and small pieces of tissue can be handled nicely with the instrument shown in the lower left corner, which takes the place of a proper section lifter and costs much less. It is a steel manicure instrument used for pushing back cuticle and cleaning the nails. One end is flat and pointed, the other broad and flat.

Lamps — Artificial illumination for visual examination and photomicrography of subjects may be provided in several ways, some of them good, others poor. Visual examination of transparent specimens may be made with ordinary room lighting, although for the higher powers

Fig. 55. Small lamp for visual examination of microscopical subjects.

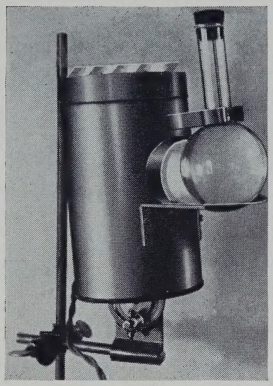


Fig. 56. High power lamp for photomicrography of microscopical subjects.

this is inadequate. General practice in laboratories is to provide individual lamps for each microscope. This affords maximum control of the light and does not interfere with other workers.

A desk light in a reflector, shown in Fig. 63, is one way to provide light for both viewing and photographing specimens. If the reflector is turned so that the light does not enter the eyes of the person using the microscope, or the camera lens, this type of lighting is quite satisfactory for less critical work. Photomicrography, however, frequently requires transmission of monocromatic light by the introduction of suitable filters, when a proper lamp housing with built-in provision for filters is superior. Such a lamp is illustrated in Fig. 55. The housing for this unit is a tin can in which sodium bicarbonate was packed for sale. The can was procured at a drug store at no cost. Fastened to the inside bottom of the can with

two machine bolts is a ten cent porcelain receptacle. Six feet of fixture wire connects the receptacle to the supply outlet. The reflector behind the bulb is a $2\frac{1}{2}$ inch watch glass. The convex surface was sprayed with aluminum paint, which provides a splendid reflecting surface. It is held to the can by a tin spider of three radial arms 120° apart. They are a quarter of an inch wide and meet at a three quarter inch disc in the center. Through this disc passes a small machine bolt that holds the entire unit to the can. The free ends of the spider are bent over to secure the reflector. The lid of the can was slit with a fine jeweler's saw. Five slits were made across the top. Each slit terminates in a short slit at right angles, reaching almost to the middle of the turned-up strips. These strips were left attached to the lid by bits of metal about one thirty-second of an inch wide. These hold the strips, which were then turned up as shown, to provide ventilation.

A fifty-watt daylight bulb was screwed into the receptacle and the center of the filament measured from the base. A hole one and one half inches in diameter was then cut in the side of the can with a dull jack knife with its center exactly in the center of the filament. A frame to hold filters or ground glass was made of thin cardboard and secured to the housing with small machine bolts, and the lamp housing was finished with a coat of black lacquer. Entire cost was about forty-five cents.

The lamp house shown in Fig. 56 is larger and slightly more elaborate, because it was made for photomicrography entirely. In order to minimize the possibility of vibration, the exposure was reduced by increasing the power of the light to 250 watts. A concentrated filament bulb was selected, with a standard screw base. The con-

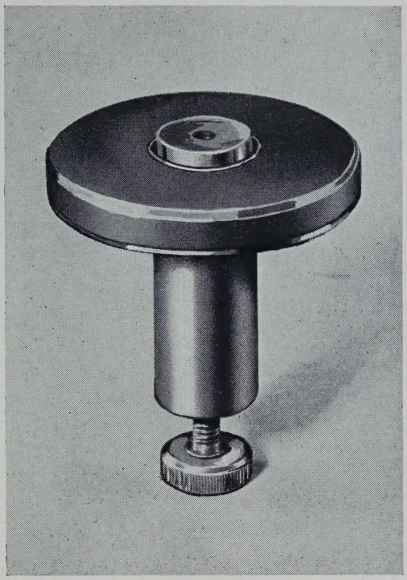
struction is the same as that of the smaller lamp house, the only difference being the shape and size of the can, which in this case is round. The exterior is provided with a small aluminum shelf and a spring clip to hold a Florence flask, which serves three purposes. First, it acts as a condenser to concentrate the light in a small spot. Secondly, being filled with water, it absorbs part of the heat from the lamp. Finally, by coloring the water with suitable dyes or chemicals, a monochromatic light may be produced. The segment of arc at the bottom, held by a wing nut, permits adjustment to any angle. The entire unit was assembled in three hours from scrap material, and cost about fifty cents, excluding bulb and flask.

SLIDES — These are inexpensive items to buy ready-made, but when making a large number of mounts the cost becomes a factor. These too may be made at home at small cost. Any photographic supply house can supply lantern slide cover glasses at about twenty cents a dozen or a dollar and seventy-five cents a gross. They measure $3\frac{1}{4}''$ by $4''$ and are cut from thin, clear glass, usually free from bubbles. To make your own slides, rule a piece of paper with a rectangle $3\frac{1}{4}''$ by $4''$. Draw a line along one long side a quarter of an inch from the edge. Now divide the remaining $3''$ by $4''$ rectangle into four equal parts, the long sides parallel with the three-inch side. Lay a lantern slide cover glass on the large rectangle and with a glass cutter scratch a line at the three-inch mark. Make similar scratches on each of the one-inch marks and break the glass. If the cutter is sharp the breaks will be true and fairly smooth. Grind the edges on a carborundum stone or fine emery paper to make them smooth. The cost per slide is thus very low and for student work the material is quite satisfactory.

SPIRIT LAMP — Several operations in preparing microscope slides require moderate heat. Bunsen burners, unless of the micro type, are too hot, so a spirit lamp or alcohol lamp is used. These may be purchased for from fifty cents to a dollar, but one can be made for fifteen cents. Most auto accessory stores and five-and-ten cent stores sell small copper oil cans for ten cents. Buy one of these and cut off the spout so that about one inch remains in the screw cap. Five cents worth of dry asbestos packing, also obtainable at the accessory store, provides the wick. A small empty cartridge-case or glass vial may be used to cap the wick and reduce evaporation.

BALSAM BOTTLE — A proper balsam container costs in the neighborhood of fifty cents. As far as results are concerned it is worth the price because it has an outside-ground stopper, a very important feature on a balsam bottle. Balsam has the nasty trick of cementing material together, so that a ground-in stopper of the reagent-bottle type would become cemented into the neck. Corks break and the bits get into the balsam and cause trouble. So we use an outside-ground stopper or cap on the balsam bottle. The cap is ground on the inside and the bottle neck on the outside. This keeps the balsam away from the two contact areas, yet makes an air-tight seal.

A most efficient substitute for a purchased balsam bottle may be had for the asking from a dentist. Many dentists make what are called inlay fillings. Instead of filling the cavity with silver-mercury amalgam they take an impression of the cavity, cast a solid metal filling from the impression and cement this in place. The cement used for this purpose is a dry powder that is mixed with metaphosphoric acid. The acid is sold in small (about one-half ounce) bottles with outside-ground caps. Ask a dentist



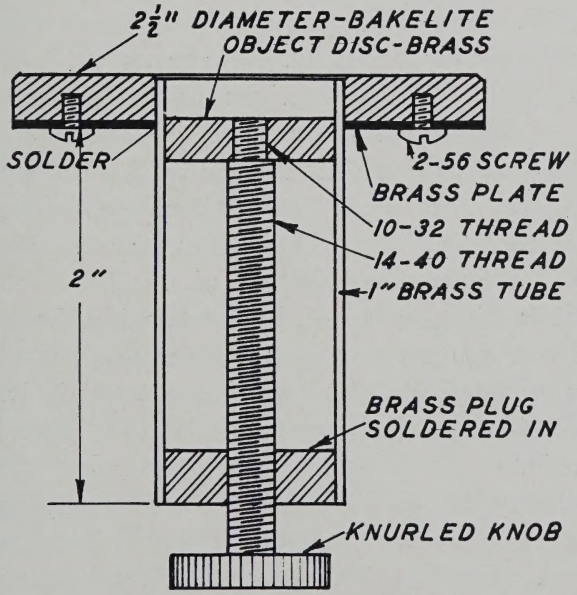


Fig. 57. Well microtome for cutting sections.

for one of these empty bottles, wash it thoroughly, dry it perfectly and you have an excellent balsam bottle.

It may be improved for this purpose by providing the cap with a dispensing rod for placing balsam on the slide. Cut a piece of small-diameter glass rod slightly longer than the distance from the inside of the cap to the inside bottom of the bottle. Fire-polish one end in the flame of the spirit lamp. Flatten the other end into a small flange by holding it in the flame until soft, then pressing it against a piece of heated metal. Coat the inside of the cap and the flange on the rod with water glass (sodium silicate). Let this dry a little and press the two together, holding them in position until the cement sets. The rod will be permanently attached to the cap, which, used as a handle, will keep the fingers free from balsam.

Microtome — The microtome was mentioned in Chapter V. This instrument is illustrated in use in Fig. 33, and as a unit in Fig. 57. Its purpose is to hold imbedded objects while thin sections are cut for mounting on a slide. It consists primarily of a tube, closed at the lower end, which is fitted with a fine-threaded screw by which the specimen is advanced between each section cut. The upper or open end is provided with a large flat plate that affords a bearing surface for the knife.

All dimensions are given in the drawing, from which the student can make or have made at small cost a similar microtome that will serve his purpose nicely. The method of using the instrument is fully described in Chapter V.

Paraffin Oven — Chapter V included numerous references to a paraffin oven, used for imbedding objects to be sectioned. Catalogs list these at prices ranging from ten dollars for the simplest oven to several hundred dol-

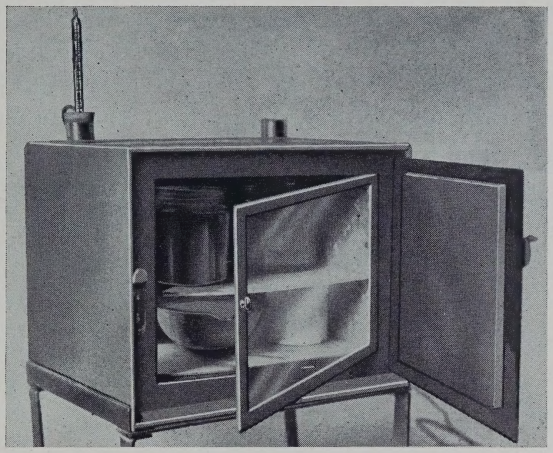


Fig. 58. Paraffin oven for paraffin imbedding.

lars for the most elaborate. This important item of equipment can be built by any tinsmith in the form shown in Fig. 58, or a substitute may be provided by the student himself. This substitute is known as a Brown University oven, because it was developed and is used extensively by Brown University students' laboratories, both as a paraffin oven and an incubator for micro-organisms.

The galvanized sheet iron oven shown in Fig. 58 was made for the author by a local tinsmith at a cost of five dollars. The inside dimensions are twelve inches wide, eight inches high and eight inches deep. It is a double-walled type of oven with a one-inch space between the walls on five sides, and has a hinged door insulated on the inside with celotex. Double walls were provided so that the space between them may be filled with water, the oven then being placed on an electric hot plate for heating, making possible its use either as a paraffin oven or an incubator for bacteria. At the rear left corner a one-inch tube is soldered into the top. This communicates with

the interior of the oven so that a thermometer may be inserted to measure the temperature. A similar tube is provided at the rear right corner for filling the space between the walls with water. The oven may be heated by using either the water jacket or an electric light bulb.

For some purposes, such as incubating bacteria cultures, the oven is used with the water jacket filled. When using it as a paraffin oven the electric bulb is used. The space between the walls is then empty, the dead-air space affording fair insulation to reduce heat-loss by radiation. The thermometer is placed in one hole of a two-hole rubber stopper, and the supply wire for the bulb goes through the other hole. The heat from a fifty-watt bulb is just enough to maintain a temperature of 56°– 58° C. By using a smaller bulb a temperature of 37°– 39° C. may be easily maintained for flattening paraffin sections. Thus a very useful accessory was provided at small cost.

The Brown University oven can be built even more cheaply. It consists of a white enamel sauce pan without a handle, a cover of sheet metal and an electric light bulb to provide heat. The cover has a hole cut in the center just large enough to permit inserting the lower part of a bulb socket. A short length of metal tubing should be soldered on some part of the cover to allow the insertion of a thermometer. The material to be imbedded may be set directly on the floor of the oven or suspended from its sides in little wire baskets hooked over the rim of the sauce pan. Changing the size of the bulb gives the required control of temperature. Since the factors of heat input and radiation are constant, the only variation in temperature will be that caused by variation in the ambient temperature. This, too, may be controlled within rather narrow limits by immersing the oven in a large pan

of water. Such an oven is very efficient and should cost less than a dollar to construct.

Even this low cost might deter some students from doing paraffin imbedding, so the suggestion illustrated in

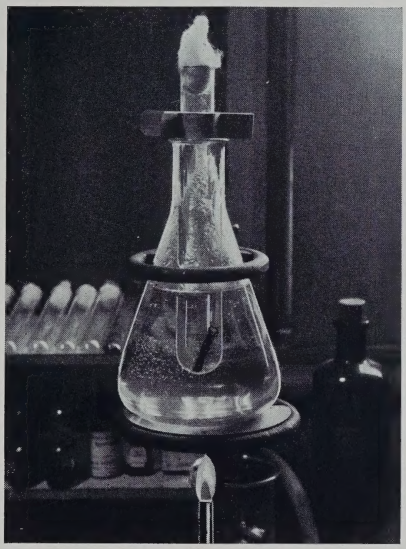


Fig. 59. Test tube and Erlenmeyer flask used for imbedding small objects in paraffin.

Fig. 59 is made. This is simply an Erlenmeyer flask containing water, and a test tube for the paraffin and the material to be imbedded. Heat is supplied by the spirit lamp which is moved toward or away from the flask as required by the temperature to be maintained. Temperature control is not very accurate, but if the paraffin is kept just above the melting point the material will not get too hot and no fear need be entertained.

TURN TABLE — This device is used for spinning rings of gold size, shellac or lacquer for shallow cells, and for

applying a finishing coat of black lacquer to the edge of the cover glass. The practice of painting a finishing ring half on the cover glass and half on the slide is rapidly losing favor except for glycerin jelly mounts. Balsam mounts are perfectly tight if made with a slight excess of balsam, as already described, and gain nothing by ringing. Glycerin jelly mounts may be made more secure by ringing with shellac or gold size.

Neat shallow cells, however, do require the use of the turn table. In its simplest form this is a block of wood, on one end of which is a circular plate free to rotate about its central axis. Provision must be made to hold the slide, or centrifugal force will throw it off the table.

To make a turn table, take a block of wood six inches long, four inches wide and seven-eighths of an inch thick. In one end, near the edge, locate a center and drill with a 3/16″ drill. Then take another piece of wood, quarter-inch plywood is excellent, and cut a disc four inches in diameter. Through the center of this disc run a 3/16″ flat-head stove bolt one inch long, with the nut on the under side. Counter-sink the top so that the screw head is flush with or slightly below the surface of the disc. The slides may be held with spring clips like those used on the microscope stage. Place the leg of the bolt in the hole in the large block. On the face of the disc rule a rectangle one by three inches in the exact center, to act as a guide in placing the slide.

To use the turn table, dip a small camels-hair brush in the gold size or shellac, start the table spinning with a quick flip and lower the tip of the brush to the slide. The result will be a circle of diameter depending upon the distance of the brush from the center of the turn table.

POLARIZER — Of the many accessories for the micro-

scope, none can compare with the polarizer for sheer beauty of results. The theory of polarized light has previously been discussed briefly, so let us examine the methods of securing polarized light at small cost.

Commercial polarizers and analyzers consist of natural crystals of Iceland spar (calcite), sawed in two longitudinally, and the two halves cemented together with Canada balsam. These crystals are called Nicol prisms, after their inventor. One of these nicols, the polarizer, is placed below the microscope stage; the other, the analyzer, is placed above the stage, between the specimen and the eye of the observer. Two types of polarizers are adaptable to student construction, one the nicol type, the other a reflecting type. The former is much more efficient, though slightly more difficult to construct, while the second type sacrifices efficiency for simplicity of construction.

Since the nicol type is the more efficient, and the difficulties of construction not insurmountable, that is the one to build. The first requisite is a pair of Iceland spar crystals, which may be procured from any house that sells mineralogical specimens to schools and collectors. When ordering them by mail be sure to specify that good, clean, clear crystals are desired. These are slightly higher in cost than less desirable specimens, but are well worth the difference. The one for the polarizer should be a rhomb measuring about one half inch square. It will be somewhat rectangular for the four side faces are never equal. The length is not very important, one half to five-eighths of an inch being ample. The analyzer crystal need be only about a quarter of an inch square and the same in length.

Having procured suitable crystals they must be sawed in half through the obtuse angles as shown in the diagram,

Fig. 60. Iceland spar is a compound of calcium, and is comparatively soft, so sawing will not be difficult. The best way to hold the crystal while sawing it is to grip it in a small vise between two pieces of thick cork, thus minimizing the danger of breaking it. This danger is very real, for while the mineral is soft it has very well defined cleavage planes along which it breaks with great ease. Be

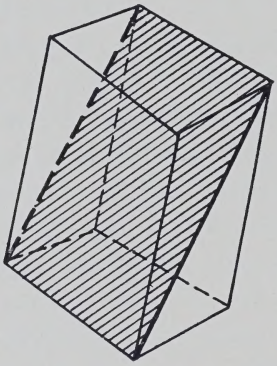


Fig. 60. Nicol prism, showing plane of section for polarizer and analyzer.

very careful while sawing to keep the saw moving in a straight line, without twisting, or the crystal will cleave and be ruined.

Sawing is done with the emery saw as described in Chapter VII. First make a deep scratch along the intended line of division with a sharp-pointed instrument, such as a heavy needle, in order to start the saw. Then use the emery saw with plenty of water. After the cut has extended to the center of the crystal it is advisable to take it out of the vise and place it upon some hard support, holding it with the fingers to complete the cut. When about a sixteenth of an inch remains to be cut proceed

very slowly, using very light pressure on the saw, for at that point the crystal is very apt to break with a ragged edge. Be warned again, to prevent cleaving keep the saw going in a straight line and do not twist or turn it. When one crystal has been sawed proceed in the same manner with the other one.

The divided crystals are now polished to a glass-like surface at each end and on the cut faces. The student may do this himself or have it done by a manufacturing optometrist. If it is done at home the faces are first ground flat on a piece of plate glass, using very fine emery powder as the abrasive, and plenty of water as the vehicle. When the faces are flat, wash plate and crystals with water to remove every bit of emery. Then continue the grinding with pulverized rotten stone. Commercial rotten stone frequently contains large particles that might scratch. To prevent this tie the powder in a piece of fine silk or bolting cloth and shake it over the glass, thus sifting out only the finest material. This grinding will produce a smooth flat face, but will not polish the crystal. The final high polish is secured by using jeweler's rouge and a piece of thin felt stretched taut over plate glass. The felt is moistened with water, the rouge rubbed into it and the polishing done by rubbing the crystal on the felt. While the work entailed is not difficult, it is somewhat tedious, and may be avoided by turning the job over to an optometrist. With his power tools he can turn out a good polishing job in a fraction of the time required to do it at home.

The polished crystals are now cemented together with some of the same balsam that is used to cement cover glasses to slides. Cement the two halves together so that they occupy the same relative positions as they did in the

whole crystal. Warm the halves slightly before applying the balsam, but do not get them too hot or the expansion will cleave the crystal. Cover the entire face with a thin film of balsam and press the two together to assure perfect adhesion at every point. The cemented nicols are now set aside for the cement to harden after which the four long sides are painted with black paint, India ink or opaque water color.

While the nicols are setting the mounts may be prepared. The polarizer prism is the most difficult to mount because this must be located under the microscope stage. The exact method of attaching it to the stage will depend upon the microscope and the amount of space available. The simplest mount is made of cardboard tubing large enough to permit insertion of the nicol. A narrow flange can be glued to one end of the tube. Thus the nicol may be attached to the stage by spring clips, or, more simply still, by strips of adhesive tape. If the microscope is equipped with a substage condenser it is an easy matter to design the nicol mount so that it slips over the condenser housing.

The analyzer may be conveniently mounted in the tube of the microscope, either in the eyepiece or lower in the tube. Whatever style of mounting is selected it is essential that one of the nicols be arranged so that it can be rotated about its long axis, for only in this way can the phenomenon of polar rotation be studied, and the gorgeous colors resulting from some materials with crossed nicols be seen. Mounting the analyzer in the eyepiece of the microscope makes this possible by simply rotating the eyepiece. If it is more convenient to mount the polarizer in a rotating mount do so, for it makes no difference which one turns.

The most gorgeous colors are produced with polarized light when a mica or selenite plate is placed in the optical path. Excellent results are obtained by using a piece of clear mica. For this purpose there is nothing better than a mica disc such as is used in a phonograph reproducing head. This is clear and unsplit, of uniform thickness, and the best mica obtainable. Discs may be purchased for a few cents at music stores or from phonograph repairmen.

From the foregoing discussion it is evident that easily procurable, low cost substitutes may be provided for the more pretentious, expensive commercial items of equipment for the pursuit of microscopy. A little thought and study is required. After all, the end is what matters, and workmen are not judged by their tools. The early investigators in microtechnique had little to work with, yet they conducted researches and made the discoveries which are the foundation of our present knowledge of science. If satisfactory results can be obtained from the use of any equipment, that equipment is serving its purpose, regardless of its appearance or origin. The greatest photographer of snowflakes who ever lived did his work with an old battle-scarred camera and an equally old lens, yet his photographs were sought after by scientists from all over the world. The point is, he knew how to get results, regardless of, or in spite of, his equipment.

Chapter XII

Photomicrography

Ever since the microscope has been in use students have made graphic records of the details it reveals. Before the invention of photography these records were drawn by hand, a laborious and often inaccurate method.

The development of photography, however, placed at the disposal of the student a rapid and accurate means of preserving in exquisite detail every feature of the object under observation.

Good photomicrographs depend upon several factors, not the least of which is good slide preparation. The camera records every detail of the slide, and if preparation is slovenly, it will present a picture of similar slipshod methods. The artist may alter his hand drawings of microscopical subjects but the camera does not have this power of elimination, therefore a more severe burden is placed upon the preparer. Unless the material has been properly prepared and mounted it is useless to expect good pictures. On the other hand, good preparations yield passable results even with mediocre microscope and camera equipment. The student should always aim to produce the best results possible with the equipment at his disposal. This will necessitate the keeping of accurate records of every exposure made. Whether the negative is good or bad does not make the slightest difference in the record, for from this record he can make the necessary alterations in conditions to improve the succeeding effort. The following table is arranged for the notation of results of photomicrographic exposures.

Record Sheet for Photomicrograph Exposures

Cat. No.	*Objective*	*Ocular*	*Mag. and Bellows Ext.*	*Condenser*	*Light*	*Exposure and Diaphragm*	*Stain*	*Filter*	*Neg. Material*	*Develop.*	*Remarks*
0.175	16 mm	× 6	× 60 – 6″	Leitz 1. 20 N.A.	250 watts critical	1/25 sec. N.A. .75	A. Hem. and eosin	K 2	Comm. Pan.	Pyro A–B–C	Good neg. Full exposure

Fig. 61.

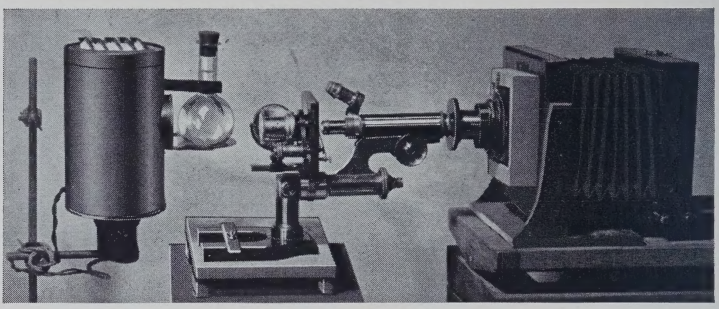


Fig. 62. Horizontal set-up for photomicrography, showing polarizer and capping analyzer in place.

Reference to the notes contained in such a tabulation will make the duplication of results more certain when dealing with similar subjects, and will indicate the changes necessary when undertaking work with other subjects. You will note that spaces are provided in the table for the name and catalog number and the stain used on the preparation. This means that when the slide was made a record was kept of each detail as noted in Chapter VIII. It would be well to review the data that the slide label and catalog should contain.

THE CAMERA — Any camera can be used for taking photomicrographs. This may seem like a broad statement, but it is literally true. The only purpose served by the camera is to hold the sensitive negative material and provide a light-tight coupling between the microscope and the negative. In its simplest form this purpose is served by a wooden box with a hole at one end for insertion of the microscope tube, and provision at the opposite end for holding the film or plate. The arrangement may be either vertical or horizontal, as best suits the convenience of the student, since either produces identical results. General preference seems to be for the horizontal

Fig. 63. Vertical set-up of camera and microscope when using a small camera.

set-up, since this permits simplified construction, greater stability, easier alignment and more flexibility. Such an arrangement is shown in Fig. 62.

It is very important that the plane of the sensitive material be exactly parallel with the plane of the slide. If this condition does not obtain, uniformly sharp negatives will be impossible. If the camera has a ground glass back for focusing, the image may be inspected visually and the alignment altered until the entire field is sharp. Roll film cameras and others not fitted with a ground glass can be aligned by measuring from the stage of the microscope to the back of the camera.

When only a few photomicrographs are to be taken at infrequent intervals it is possible to secure satisfactory results by merely standing the camera on the eyepiece of the microscope as shown in Fig. 63. This arrangement is quite feasible if the construction of the camera is such that it will rest firmly on the eyepiece. This condition is fulfilled in most box cameras of the Brownie type, a great many of the small folding cameras and some of the miniature cameras. Those types which will not balance in the position suggested must be supported in some way to prevent falling. Lightweight cameras with a tripod socket can be supported very nicely on a chemical ring-stand by making an adapter with a projecting screw that engages the tripod socket, while a clamp holds it to the vertical rod. The microscope then stands between the legs of the tripod. The ingenious student will be able to devise some means of holding the camera in place on the microscope.

Bear in mind three things. First, the lens mount of the camera must be axially central with the microscope. Secondly, the front of the camera must be as close as possible to the eyepiece of the microscope to reduce the possibility of cutting off the edges of the circular field, and the joint must be light-tight. Finally, the entire outfit must be set up in a place reasonably free from vibration. If a concrete basement floor is available that is an excellent place for the work. Be sure the table on which the outfit stands is resting solidly on the floor. If one leg is short, block it up with pieces of cardboard to insure a firm foundation. The results attending such an arrangement are not the best, naturally, but careful work will produce acceptable results.

If the student wants to improve his work and get the

best pictures his equipment is able to produce, his preparations will need to be somewhat more elaborate. For this purpose, and especially for work at high powers, the horizontal arrangement is far better. Great flexibility in the disposal of equipment is one of its outstanding features, as well as firm construction and ease of manipulation. The floor space required is greater than in the vertical type, but this disadvantage is more than offset by the numerous advantages.

All professional and some of the amateur microscope stands have a knee joint that permits inclination of the tube to an angle of 90 degrees with the base, thus making the tube horizontal. When used in this position the microscope is set on a bench or table, the camera attached to the eyepiece and the illuminant placed behind the stage, all in a straight line, thus making for the greatest efficiency. Into the light path we can then introduce condensing lenses and diaphragms to parallelize the beam, and filters to control its color.

ADJUSTING THE APPARATUS — Set up the camera, microscope and light, fastening each rigidly in place. Place the slide on the stage and focus it roughly, either with the eyepiece or on the ground glass. If the microscope is being used without the eyepiece you will probably see on the ground glass a number of curious reflections. Examine the connection between camera and microscope. If this is light-tight remove the ground glass and look down the microscope tube. The cause will at once be apparent. The inside of the tube is shiny. In this case make a tube of black velvet to line the draw tube, and the trouble will be corrected.

Remember that when making photomicrographs, errors of focusing cannot be corrected by stopping down the

lens, as is sometimes done with photographic lenses. The focus must be exactly right on the ground glass for critical definition. To assist in securing the best possible focus, the ground glass should have a clear spot. Rule fine diagonal lines from each corner of the glass with India ink and a ruling pen. Over their intersection cement a cover glass with Canada balsam. Exact focus can then be secured by examining the image with a magnifier adjusted so that it is in exact focus on the cross lines. When the image and the lines are both in focus the definition is the best possible.

In order to take full advantage of the resolving power of the objective certain conditions of illumination must be fulfilled. These are not difficult to attain at low powers, but when lenses of wide numerical aperture are used the conditions are more difficult to meet. Full resolving power is secured only when (1) the entire aperture of the objective is filled with light, which may be ascertained by looking down the tube with the eyepiece removed, and (2) when critical illumination is used.

Critical illumination is obtained, theoretically, when all the light waves reaching the slide and forming the image at any one instant of time also leave the light source at the same instant of time and come from the same point. This condition is secured in practice by focusing the light source on the object by means of condensing lenses. These lenses, with a parallelizing diaphragm, are used between the light source and the substage condenser. The flask shown on the lamp house (Fig. 56) serves as one lens, auxiliary condensing lenses such as a plano-convex or a double convex lens in an independent mount as the other. Between the two lenses is placed a diaphragm, either of the iris type or a plate with several holes of

graded diameters. The focal length and placing of the lenses determine the size of the image of the light source projected on the substage condenser.

After the light source has been arranged in line, close the iris of the substage condenser to its smallest diameter and focus this opening accurately on the ground glass, centering it carefully. Now open the substage iris and cut down the iris on the outside condensing lens. Move the parallelizer back and forth until its image is sharp on the ground glass, then center it and lock in place. Close the substage iris again and adjust the position of the light source so that its image is sharply defined and falls centrally on the substage iris. This light image should just fill the working aperture of the objective.

One of the most frequent causes of poor images in photomicrographs is the diffraction caused by the use of too much uncontrolled light. Only that light that passes through the objective is of any use in forming the image, hence only that portion of the slide directly under the objective should be illuminated. To secure this result the substage iris must be reduced to the absolute minimum for the objective in use. Examine the image and while watching it, reduce the iris opening until the scattered light disappears and the image is sharp and clean. Do not use an opening smaller than is necessary, for this too results in degraded images.

With the object sharply focused examine it on the ground glass to determine whether the size is correct. This can be varied at will by moving the ground glass toward or away from the eyepiece. The magnification of microscope optical systems for photomicrography is usually computed at a point eight or ten inches from the eyepiece. Thus, if a X100 objective is used with a X6 eye-

piece the magnification represented by the photograph will be 100 x 6 or X600 with a bellows extension of eight inches. If the bellows is extended to sixteen inches the magnification will be doubled and the photograph will show a magnification of X1200. The resolving power, however, will not be increased. The apparatus is set up and adjusted and we are now ready to make the negative.

Since the only reason for making photomicrographs is to record the details of the object, the matter resolves itself into a recording of contrasts. This is not always easy to do, for the contrast of different subjects varies greatly. The most difficult cases encountered are those arising from a lack of contrast between the object and the background, as in lightly stained sections, in many aquatic organisms, micro-organisms and the like. Sections that are deeply stained with haematoxylin, eosin, indulin, safranin, etc. usually present little difficulty when photographed as a whole on a clear ground. When only a small area is to be photographed at high powers to show structural details, trouble may be encountered.

Dirt in the form of dust and grease on the lenses may ruin otherwise contrasty images. Lenses should be carefully cleaned with lens paper, or the dust accumulation will cover the image with scattered light and ruin the contrast. Diffraction resulting from the passage of extraneous light through an unnecessarily large parallelizer or substage iris will destroy contrast. Control of contrast lies in the choice of the proper filter to use with color sensitive material to record the image. Filters are used to produce monochromatic light, by which the details of microscopic structures may be intensified or reduced at will.

In order to give the student a clearer understanding of

the nature of light and color, let us examine these two phenomena.

If a beam of light, either daylight or artificial, is analyzed in the spectroscope, it will be found to consist of several bands of color, called the spectrum. There are three major divisions of color, blue-violet, green and red, with intermediate steps that form the transition bands. According to the absorption theory of color formation, color is the sensation produced when white light falls upon an object which does not reflect all of the incident white light. Thus, if white light falls upon an object that absorbs the entire spectrum except the green portion, that portion is reflected to the eye and we see green in the object. In other words, a colored object is one which does not absorb all the constituents of white light, but reflects some, the reflected portion producing the sensation of color.

Again using the spectroscope, examine the light reflected from a colored object or passed through a colored filter. We find that instead of the continuous spectrum of white light we now have a spectrum from which a part of the band is missing. In the spectroscope the missing portion appears as a black line or band which represents the color that has been absorbed by the filter or reflected by the colored object. This is known as the absorption band. Since reflected light produces the sensation of color in an object, it follows that the object is absorbing the complementary or reverse color component of white light.

Turning the spectroscope upon objects of varied colors we find the absorption band shifting with each change of color. We find, for example, that a light blue object has an absorption band in the red portion of the spectrum. Since light blue is a mixture of green and blue-violet light,

of which red is the complementary color, the blue sensation is due to the absorption by the object of all the red portion of the incident white light, and its reflection of the green and blue-violet components. Similarly, a sensation of yellow color results when an object absorbs the blue-violet portion of the spectrum and reflects a mixture of green and red light.

Now the question arises how this color theory may be used in photomicrography. The answer is that by intelligent selection of a filter we can increase or decrease the contrast of the object and its background or the various details within the object itself. In order to make this selection with precision it will be necessary to go deeper into the scientific theory of color.

Light propagation is in the form of waves, each color having a specific wavelength, measured from the crest of one wave to that of the next. This distance is expressed in Ångström units, one unit being one ten-millionth of a millimeter. By assigning each color to its proper place and expressing this position in terms of Ångström units we are able to place the absorption band. With this information and a knowledge of the absorption band of the filter we can accurately forecast the effect.

From the following diagram we see that the blue-violet band is at the left of the spectrum with a wavelength of 4000 units and extends almost to the blue-green which occurs at the 5000 unit division. Bright green then extends to about 5500 units, orange and yellow to 6000 units

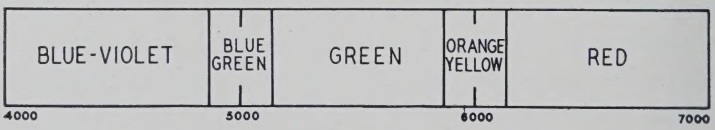


Fig. 64. Diagram of absorption band.

and red completes the visible spectrum, extending to 7000 units. Thus the position of an absorption band may be accurately placed by referring to it in terms of Ångström units. Suppose we say that the absorption band of a filter lies in that portion of the scale from 6000 to 6400 units. We know at once that this filter is absorbing the red waves that lie in that portion of the spectrum and is passing the blue-violet and green light.

We may also approach this subject by thinking in terms of the colors remaining after the filter has absorbed some of the components of white light. These are called the residual colors. This method is best illustrated by another diagram, in which the absorption bands are shown with sharply defined edges. This condition does not always exist in practice, especially with natural colors, but most microscope stains and filter stains are rather sharp, justifying our assumption of a sharp absorption band for this purpose.

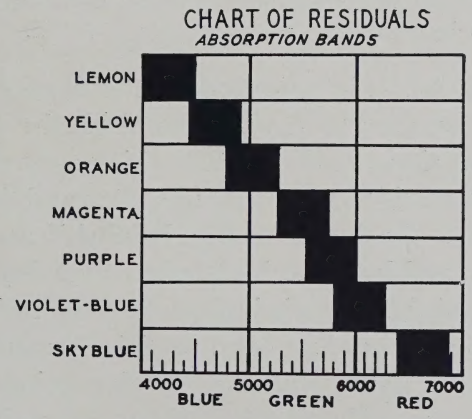


Fig. 65. Diagram of the residual colors left in the spectrum after certain bands have been absorbed.

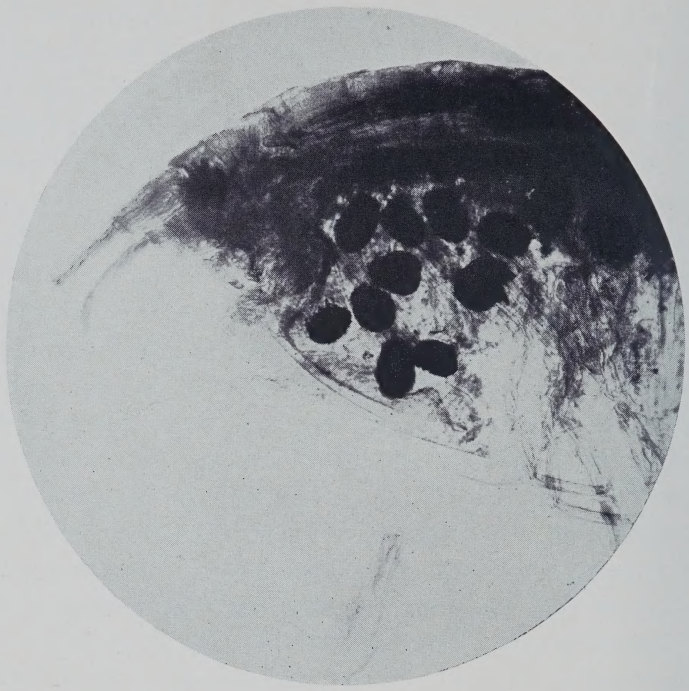


Fig. 66. Fresh water crustacean photographed for contrast with background. Note absence of detail. X25

The diagram (Fig. 65) shows an absorption band extending from 4000 to 4400. The residual color here is light yellow, since the filter is absorbing most of the blue-violet light and is transmitting the green and the red, and a little blue. As the absorption band shifts to the right more violet and less blue is absorbed, and along with the blue some of the green is included, so the residual color is yellow of a deeper hue because of the presence of more violet light. Again shifting the absorption band, we eliminate practically all of the blue-green, leaving the deep violet, bright green and red, which produces a residual color of orange. If we eliminate most of the green

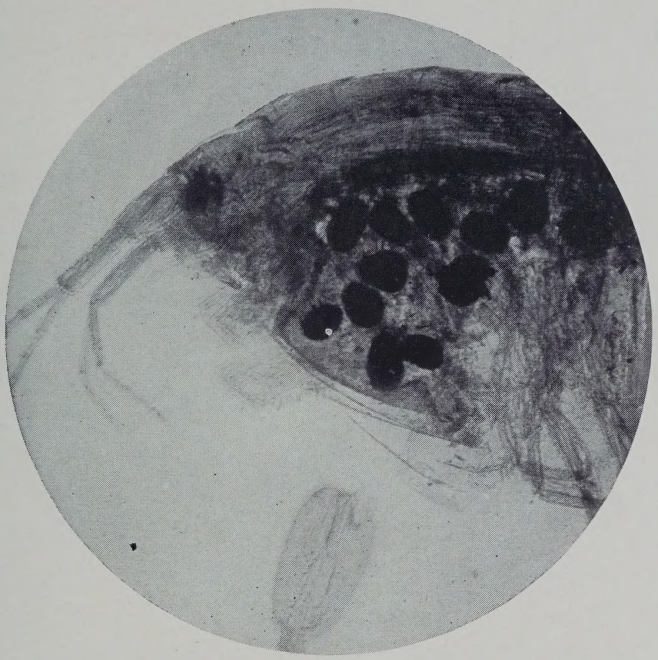


Fig. 67. The same crustacean photographed for detail. The dark objects in the thorax are eggs.

by moving the absorption band from 5200 to 5600 we will have a residual of violet, blue-green and red, which produces a magenta or red-purple. When the band shifts to the region from 5600 to 6000 most of the green and orange-yellow are absorbed, leaving a residual of violet-blue. Another shift of the band to a position from 6400 to 6900 stops all red waves and allows the transmission of the blue-green, the green and a few of the orange-red to produce a light blue color.

We are now in a position to select a filter which we are sure will produce the desired result. If we wish to make a color as dark as possible we select a filter whose transmission band completely covers the absorption band of

Fig. 68. Blue cobalt mercuric thiocyanate crystals as seen through the microscope. X125. Courtesy of Eastman Kodak Co.

the color to be photographed. In other words, it must be photographed by the light of the wavelengths that make up its absorption band.

If we wish to secure the maximum amount of contrast between a stained subject and the background we use panchromatic negative material and a filter having the same absorption band as the object itself. For example, let us suppose we have a section stained with eosin to be photographed with the maximum of contrast with the background. Eosin absorbs light at from 4900 to 5300 units. If we use a filter that transmits light at from 5000 to 5400 units the resultant photograph will show the section in high contrast, black and worthless because of

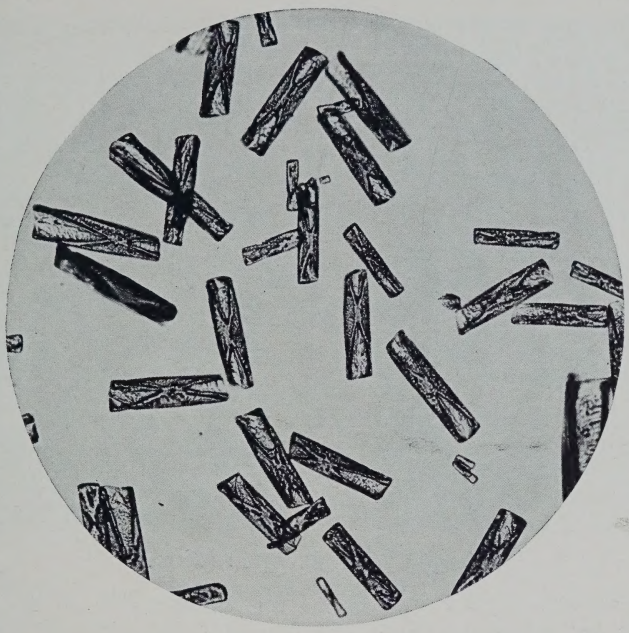


Fig. 69. Same crystals photographed on Eastman "M" plate with C filter. Courtesy of Eastman Kodak Co.

blocked up detail. This filter is greenish-blue, the complementary of red. Now going to the other extreme, let us photograph our section by light of 6300 to 6500 wavelengths, the same as transmitted by eosin. The result will be a thin, flat negative, containing little detail. Somewhere between these extremes must be a medium that will give the desired result. This is to be found at the border of the absorption band of eosin, so if we photograph by light of wavelength 5700 we will get a negative of normal contrast, full of detail and entirely satisfactory. This filter is greenish-yellow in color.

This brings us to the next consideration, that of contrast in the object itself. It is necessary to record the

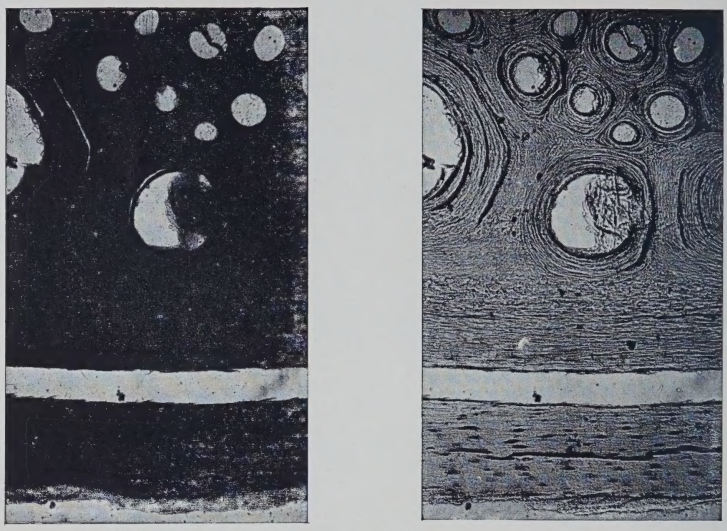


Fig. 70. Whalebone section.

Photographed with blue filter for maximum contrast.

Photographed with red filter for maximum detail.

Courtesy of Eastman Kodak Co.

details of various structural elements in contrast with one another in order that their distinguishing characteristics may be delineated. To do this the usual procedure is to photograph the object by the light it transmits. Thus, if the beetle *Sylvanus surinamensis* (Fig. 46), which is brownish-yellow in color (wavelength 5800 to 6100) were to be photographed for contrast within the object, we would use light of the same wavelength, employing a yellow or red filter and a panchromatic plate. If we desired contrast with the background we would make the photograph using a blue filter in the absorption band. This would render the insect black and devoid of detail.

Filters may be used singly or in combination. Which

filter or which group to be used in each case is decided by the operator. One way to decide is to make the selection by the methods given above; the other is by visual examination of the subject through the microscope with different combinations of filters until one is found which gives the desired results. Due to the great variation of color sensitivity in different eyes the former method is preferred since it is predicated on scientific facts. Manufacturers of reliable filters will provide technical data on absorption bands and transmission bands. Corresponding data for microscope stains may be secured from manufacturers of stains. For the student's convenience the following table of stains and their absorption bands is included. This contains only the stains used in making preparations described in this book.

Stain	Color	Absorption Band	Band Used
Aniline blue	Blue	5500–6200	5600–6000
Eosin	Red	4900–5300	5100–5400
Fuchsin	Red	5300–5700	5100–6000
Haematoxylin	Dark blue	Gradual through green	5100–6000
Methylene blue	Light blue	6000–6200 & 6500–6800	6400–6800
Methyl violet	Deep violet	5800–6000	5600–6000
Methyl green	Blue-green	6200–6500	6100–6800
Picro-carmine	Red	5100–5300 & 5600–5700	5100–5400

Unstained sections, unstained whole objects of low contrast, such as aquatic forms, diatoms, colorless insects, etc. should be photographed in polarized light with crossed nicols (applicable only where objects are optically active), on a dark-field or with negative material of high contrast. This is referred to by the manufacturers as process material, and for greatest contrast should be developed in a caustic-hydroquinone developer.

Low-contrast objects may be advantageously photo-

graphed by using an oblique light. While this is somewhat analogous to dark-field illumination it differs in that

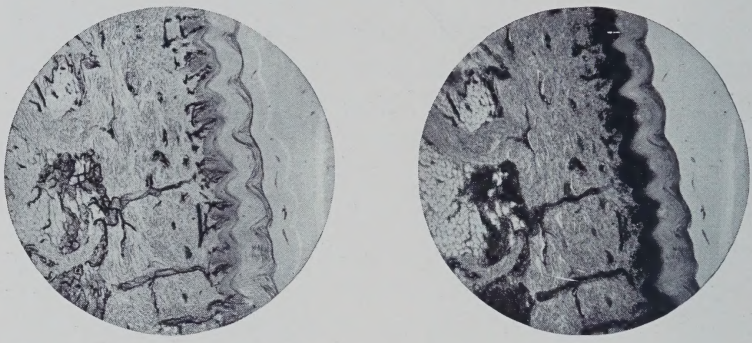


Fig. 71. Section of skin of man. X13

With dye injected blood vessels. Wratten "M" plate and F filter.

Wratten "M" plate and B filter.

Courtesy of Eastman Kodak Co.

the object in a true dark-field illumination is uniformly lighted from all sides by the dark-field condenser, while in oblique lighting the specimen is lighted only from the mirror side. This is often an advantage since it gives the object a high-light side that seems to add a third dimension, making it appear somewhat stereoscopic.

Illumination is achieved by swinging the mirror on its arm until it is close to the underside of the stage, and tilting it so that it projects a beam of light diagonally across the underside of the slide, rather than vertically through it. The contrast produced by this lighting can be further increased by standing the microscope on a piece of black velvet, or, better still, by arranging a piece of black velvet under the stage in such a way that it does not interfere with the light beam. This diagonal lighting

Fig. 72. *Sylvanus surinamensis,* photographed with diagonal lighting to show high-light effect on partial dark-field.

is reflected from the original light source, so that the in-line system of illumination cannot be employed.

Calculating the Exposure — The most satisfactory method of arriving at the correct exposure is to make a set of test negatives of an average slide under standard conditions. Future exposures may then be calculated with reasonable accuracy by referring to the record. Variable elements must be reduced to a minimum to cut down the chances of error. We standardize the light by using the same bulb and by placing the microscope, condenser, parallelizing iris and lamp house in the same rela-

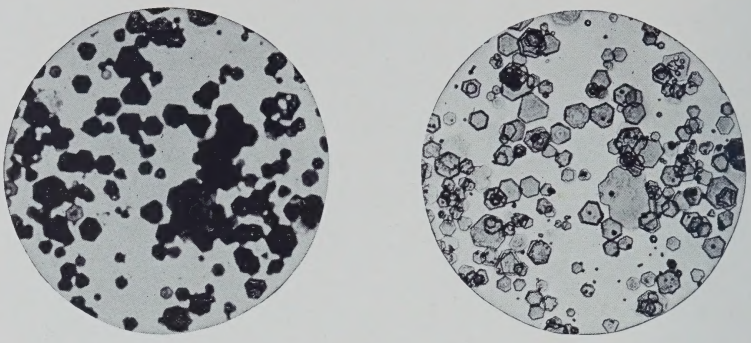


Fig. 73. Lead iodide precipitate. X125

On ordinary plate. On " M " plate with B filter.

Courtesy of Eastman Kodak Co.

tive position. Variables of magnification factors and bellows extension affect the exposure in mathematically exact ratios. Data on variables of filter factors and speed of negative material are available from the manufacturer.

Let us first examine the effect of numerical aperture on exposure. It is an optical rule that exposure varies as $\frac{I}{(N.A.)^2}$. This may be tabulated as follows:

TABLE OF NUMERICAL APERTURE FACTORS

Focal Length				*Average N. A.*		*Approx. Exposure Factor*
4	inches	100	mm.	.08		40
3	"	75	"	.09		30
2	"	50	"	.15		10
1	"	25	"	.25		4
2/3	"	16	"	.35		2
1/2	"	12	"	.45		1¼
1/3	"	8	"	.50		1
1/4	"	6	"	.8		2/5
1/6	"	4	"	.85		1/3
1/8	"	3	"	.9		1/3
1/12	" (Oil Imm.)	2	"	1.3	(only 1.0 for photo.)	1/4

This table is for professional microscopes and does not apply to amateur equipment. If the student is working with a microscope in which the optical system is designated in terms of magnification, the following table will give the data necessary for computation of exposures. Given the correct exposure without a filter on given negative material at a given magnification, the factor for any other magnification may be found in the table, since exposure varies directly as the square of the magnification.

TABLE OF MAGNIFICATION FACTORS

Magnification	*Exposure Factor*
10	1/100
25	1/16
50	1/4
100	1
250	6
500	25
1000	100

Thus, if correct exposure with standard lighting is found to be one second at X100, the correct exposure under exactly similar conditions will be six and one quarter seconds at X250, since $250^2 = 6.25$. Six seconds is close enough for practical work.

Exposure varies with the bellows extension in the same ratio as magnification, since it is, in effect, the same thing. As the ground glass recedes from the eyepiece the image grows larger and less bright, for the same amount of light is now being used to cover an increased area. For each doubling of the bellows extension the exposure must be squared. For example, if the correct exposure with six inches of bellows is two seconds, the exposure for twelve inches of bellows is four seconds, for twenty-four inches, sixteen seconds, etc.

We have stated that manufacturers of sensitive material and filters will supply tables of comparative speeds and multiplying factors for filters. Both of these sets of data are necessary in the calculation of exposure. Suppose the test negatives upon which future exposures are to be based were made on commercial panchromatic film without a filter. Using the same film we now want to know how much the exposure must be increased if we use a red filter. Reference to the table tells us that the exposure must be increased ten times to secure a negative of the same density. Now let us assume that the commercial emulsion is not contrasty enough, so we decide to use process panchromatic film with a red filter. Reference to the table of comparative film speeds informs us that process film is eight times slower than commercial panchromatic film, the filter factor remaining the same. So, with process film we multiply our first exposure by eight and this result by ten to get the correct exposure.

Before any of this data can be used for computation of subsequent exposures, test negatives must be made under standard conditions. This can be done by taking an objective, say N.A. 0.50, and making a photomicrograph at X100 without a filter. The correct way to expose the test negative is to withdraw the dark slide and expose it for a time estimated to be nearly right, say one second. Then insert the slide a quarter of the way and make another exposure of one second. Push the slide in to the halfway mark and expose for two seconds, push it in three quarters of the way and expose four seconds. The first quarter of the negative will then have had one second exposure, the second quarter two seconds, the third quarter four seconds and the fourth quarter eight seconds. Some effort should be made to estimate the correct ex-

posure as a starting point. If the correct exposure is in the neighborhood of 1/25 of a second, one second, the lowest step in the test, would be too much.

Now let us compute the exposure from the beginning. We have to photograph a section with a 16 mm objective, N.A. 0.25 at fifty diameters on commercial panchromatic material, using an orange and a green filter. The following calculation, based on test negatives made under standard conditions, will give the correct exposure.

Standard exposure x Factor for N.A. x Magnification Factor x Filter Factor

Suppose our test negative made under standard conditions shows an exposure of one-half second to be correct. We then calculate as follows:

.5 seconds x 4 x 1 = 2 seconds
Filter Factors — green = 10 seconds
orange = 4 seconds
∴ 10 x 4 = 40 seconds
Hence 2 sec. x 40 sec. = 80 seconds exposure required.

When using an amateur microscope for which the N.A. is not known, proceed as above, omitting the second (numerical aperture) factor.

As the negative material and bellows extension are altered the proper multiplying factors must be included in the calculation.

It is assumed that anyone sufficiently advanced in microscopy to take up the making of photomicrographs is also competent to do his own negative processing, so we will omit the purely photographic aspects of the operation.

Since this book is intended for the novice as well as the more advanced student, it might be well to amplify an

earlier statement, to the effect that any camera can be used for taking photomicrographs.

The author recently saw a very clever device made by a student using a No. 2 Brownie box camera with a microscope. The camera did not balance safely on the eyepiece of his amateur microscope, so he made a block of quarter inch wood slightly larger than the front of the camera. In this he cut a hole just large enough to fit over the eyepiece and lined it with black velvet. To one face of the block he glued another block of the same size with a hole in it slightly smaller than the eyepiece housing. The two blocks formed a rebated hole that fitted snugly over the microscope. To the upper face of the block he tacked a short tin tube of such diameter that when the outside was covered with black velvet it fitted into the lens opening of the camera. He thereby provided a solid support for the camera and a light tight coupling to the microscope.

When using box cameras the lens must be left in place, unless the camera is to be used for no other purpose than photomicrography. The photographic lens has no effect upon the image produced by the microscope. Removable lenses should be screwed out of the shutter or barrel. The usual amateur practice is to set the shutter for time exposure, open it and make the exposure by turning the light on and off by a remote switch to prevent vibration.

Photomicrographs Without the Eyepiece — When photographing entire specimens or large sections it sometime happens that the subject cannot be covered entirely by a low power objective. Such cases may be dealt with by removing the eyepiece from the tube and using the objective alone. This enables us to include a larger field, although the magnification is not as great. Unless the

objective is very good the edges of the field will not be sharp. The marginal definition can be improved by inserting a black paper diaphragm with a small hole in its center in the objective tube. The edges of the hole must be clean and sharp, such as are made by small paper-punches. Draw the outline of the disc with a compass to locate the center, then cut it to fit the tube snugly and punch the hole in the exact center. Push the diaphragm into the tube, being careful not to scratch the lens.

For this kind of photography the light can be considerably less than that required for regular work. It must be just as carefully arranged, however, for the effect of diffraction is increased in wide fields, because of the larger area of illumination. The inside of the microscope tube should be lined with black velvet to avoid reflections that would cause flare spots. With the change in illumination

Fig. 74. Flea from cat. This photomicrograph shows the opacity resulting from incomplete dehydration. Irving L. Shaw.

the exposure will be altered so that a new test negative will be needed.

PHOTOGRAPHING OPAQUE OBJECTS — The technique of the photography of opaque objects differs from that of transparent objects principally in the lighting. Illumination for opaque objects must come from above the object. For this purpose the large microscope light described in Chapter XI is excellent, all of the illustrations of opaque objects in this book having been made with it. The condensing flask on this light makes it possible to concentrate

Fig. 75. Photomicrograph of smooth paper surface. The black lines are ink lines drawn with a ruling pen. Extremely oblique light used to give relief. X100

the light in a spot that can be placed exactly where it is needed. At the same time the lamp house itself is far enough away from the object not to interfere with the camera when working close to the subject.

When making enlarged photographs directly from the

Fig. 76. Eggs of *Podisus spinosus.* Photographed to show markings on caps. X75

subject several courses are open. The entire microscope may be used, or the microscope stand and the objective only. The objective may be removed from the stand and used to replace the camera lens, or a short focus photographic objective may be used in a camera of long bellows extension.

Subjects having practically all the desired detail in the same plane may be satisfactorily photographed by the first method. The depth of focus of microscopic objectives of even medium power is very small, so that unless the surface of the object is almost flat a great part of the detail will be out of focus.

The photograph of the surface of a smooth pen-and-ink board shown in Fig. 75 is an illustration.

The second method is used when not so much enlargement and more depth of focus is required. The illustration showing the markings on the caps of the eggs (Fig.

76) was made by this method. It will be observed that the depth of focus is somewhat increased, including the entire depth of the cap, which is about 1/100 of an inch, with sufficient definition.

Method number three was used to make the negative for Fig. 77.

The same eggs shown in the last figure were photographed with a 32 mm. objective held in the iris diaphragm of an old lens barrel fastened to the front of the

Fig. 77. Eggs of *Podisus spinosus*. Photographed to show entire depth of specimen. X30

camera. A diaphragm of black paper was inserted in the tube to increase the marginal definition and depth of focus. The bellows extension was 12 inches. You will note that the depth is considerably greater, since both the caps and the bottom of the open eggs are in focus. The magnification is not as great. These eggs are about 1/64 of an inch high, which gives some idea of the depth of focus.

Many subjects may be photographed only by the fourth

Fig. 78. Head of spider. Photographed to include as much depth as possible.

method. A short-focus photographic objective with an iris diaphragm is used with a long bellows. The iris diaphragm increasing the depth of focus is an advantage. The illustration (Fig. 78) was made with an *f*:3.5 ciné lens held in an iris lens flange on the camera. The thirty-six inch bellows could not be entirely used because the lens approached the subject too closely to permit proper illumination.

The greatest difficulty encountered in photographing opaque objects is the lighting. When using a long-focus micro-objective in the draw tube, either with or without an ocular, the separation between subject and objective is usually great enough to permit projection of a spot from the lamp onto the subject. When working with a photographic lens this is so close to the subject that proper lighting is difficult. Various expedients, some of them calling

for considerable ingenuity, are used to secure the desired results.

The usual practice of the writer is to illuminate from one side with the projector-lamp, focusing to a small spot. This is placed so that it strikes the subject at an angle of about 45 degrees from the top and front. An auxiliary light is then directed at the shadow side by picking up the light from a 100-watt clear bulb on a concave mirror, focusing this in another spot on the subject. The principle is exactly the same as that used in ordinary lighting for portrait photography, and achieves the same result. If a uniformly strong light were used on both sides of the subject the modeling would be destroyed and there would be no sense of roundness. If a ventral light is required to illuminate dark places on the under side of the subject it is a good plan to place the subject on a sheet of glass, under which is a light so controlled that only the immediate area of the subject is illuminated. Otherwise there might be too much light, resulting in flare or halo.

Some time ago the writer had to photograph a number of opaque objects which required even lighting over the entire area. Detail, not modeling, was important. The depth of focus required the use of a photographic objective and iris diaphragm, so the ciné lens was used. The lighting problem was solved by making a square frame of wood to fit over the frontboard of the camera, the lens projecting through the frame. A 32-candle-power auto headlight bulb in a reflector made from a smooth gelatin dessert mould was placed at each corner of the frame. The four bulbs were wired parallel and operated from a toy train transformer. This arrangement provided a perfectly flat, even light that recorded details very well.

STEREOSCOPIC PHOTOMICROGRAPHY — Stereoscopic pho-

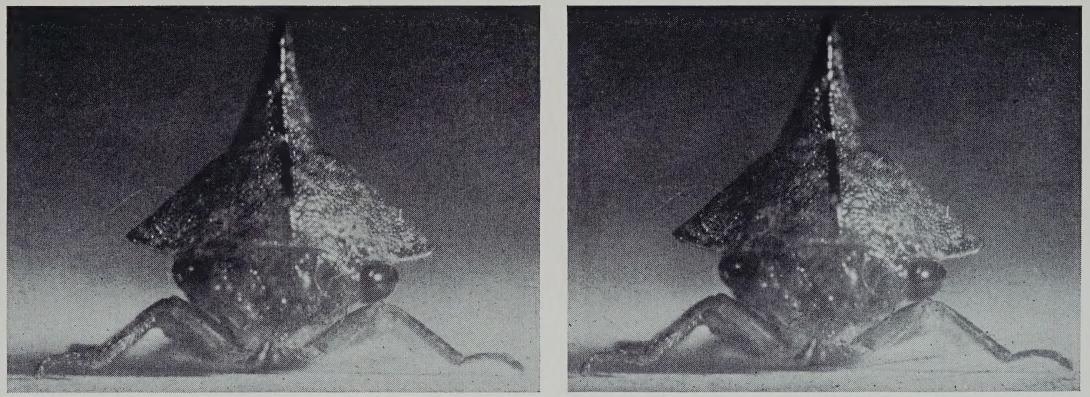


Fig. 79. Tree-hopper, *Telamona* Sp., photographed in stereo by moving the camera.

tographs of microscopic subjects yield good results and make excellent study material. The process is fully adaptable only to opaque material, such as is illustrated in Fig. 79. Sections are so thin that little would be gained by making them in stereo: insects, coins, leaves, fabrics, crystals, etc. make excellent stereo pictures.

The easiest and most precise method is the one described above, using a photographic lens on the camera which must be mounted on a platform so that lateral motion of the entire camera is possible. The negative holder is provided with an extra dark slide that uncovers only half of the plate at one time. This may be made of heavy cardboard cut to a size to fit the frame of the holder tightly.

The subject is set up and lighted as described above, and is then focused so that its center comes well off to the side of the center of the ground glass, let us say the left side. Its center should be about one and a half inches from the center of the glass. Cover the right side of the holder with the half-slide and make the exposure. Remove the holder and slide the camera to the right so that the image now moves to the right side of the center, placing it in the same relation as the first image. Cover the left side of the negative and expose again, giving it exactly the same time as the first exposure.

The prints from these negatives must be transposed, that is, the left hand negative, looked at from the emulsion side, right side up, must be printed on the right hand side of the sheet, and the right hand image on the left side of the sheet. The easiest way to do this is to make the prints on separate pieces of paper, marking them left and right, and then mount them in their proper places on a card mount.

Another method of making stereo negatives does not involve moving the camera, but moves the subject, so that the displacement on the ground glass is about three inches. While this method is effective there is danger of shifting the subject so that the two images will not coincide when viewed through the stereoscope.

This treatise of photomicrography contains sufficient information for the beginner to enable him to produce satisfactory photomicrographs from the material he collects. The author has endeavored to give information and data to assist the student in shaping his course along the approved lines of practice. If specific information and data on any branch of the work are required they may be secured by referring to the books mentioned in the bibliography.

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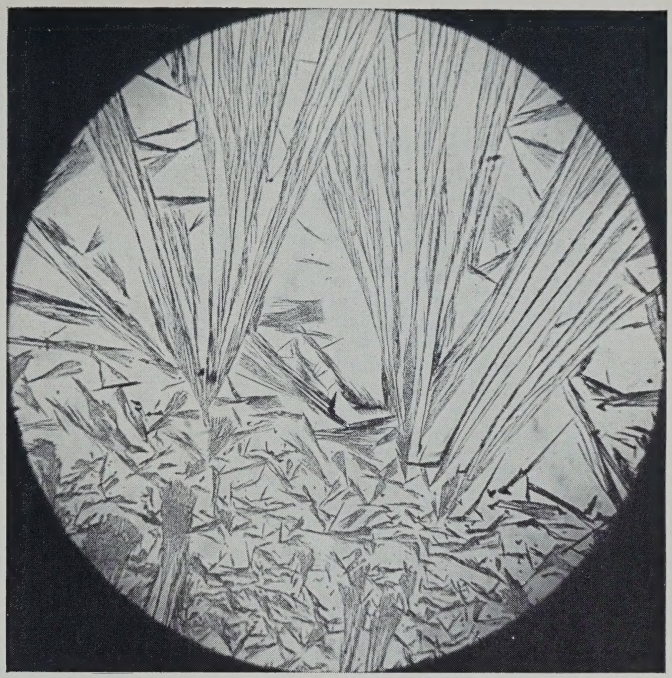

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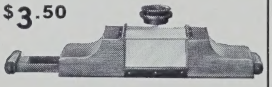

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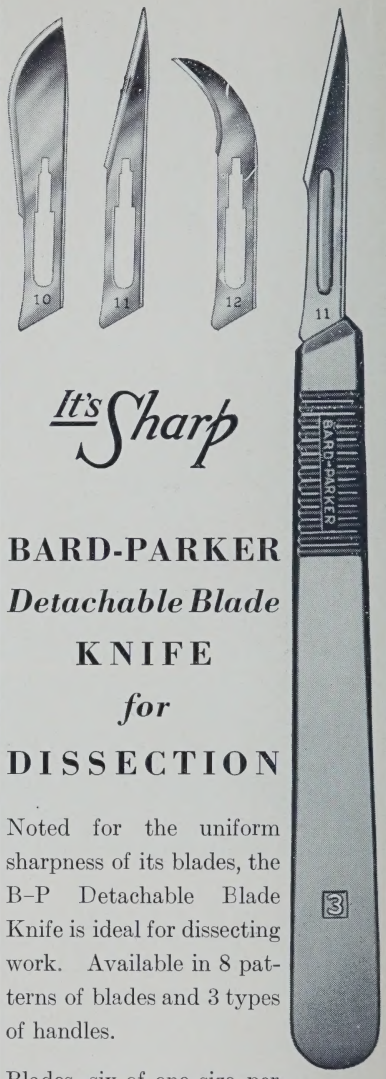

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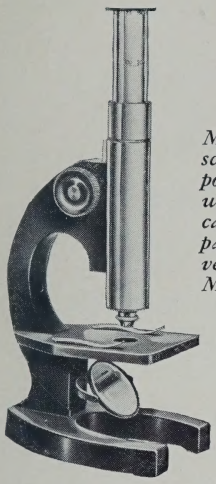

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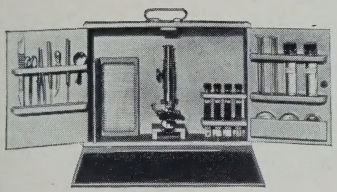

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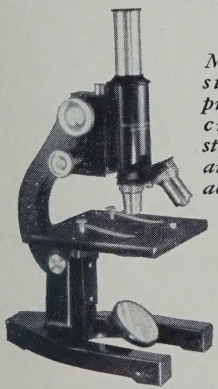

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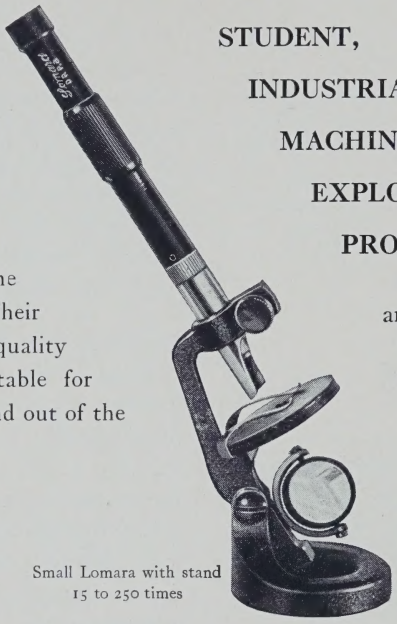

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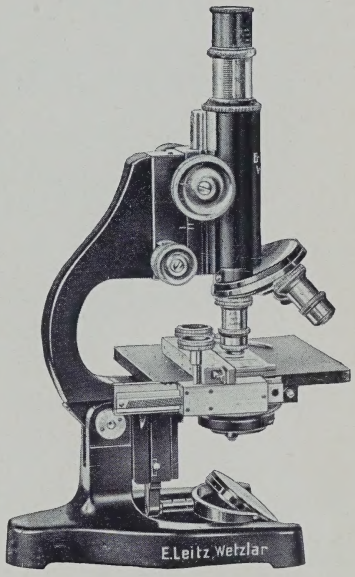

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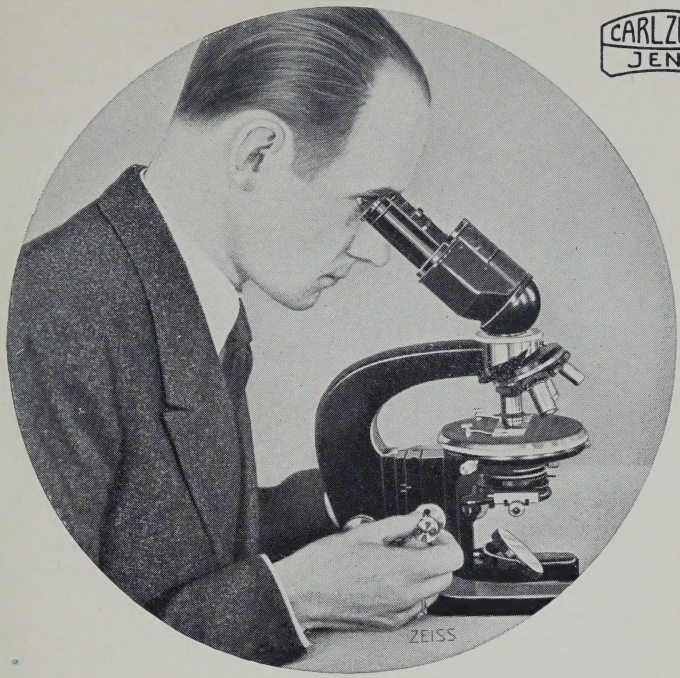

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